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(54) Title: METHOD OF SCREENING FOR NEUROPHARMACEUTICALS USING THE *DROSOPHILA* GENE *VOLADO* AND ITS MUTANTS

(57) Abstract

A new memory factor *Volado* is described along with the corresponding gene, protein sequences, and two mutants identified as $Vol^1$ and $Vol^2$. The locus encodes two isoforms of a novel α–integrin expressed preferentially in mushroom body cells. *Volado* mutants display an impairment of olfactory memories within 3 minutes after training, indicating an essential role for the integrin in short–term memory processes. Conditional expression of a Volado transgene during adulthood rescues the memory impairment. This rescue of memory is reversible, fading over time along with expression of the transgene. The present invention provides a novel method for screening for cognitive enhancers using the *volado* and integrin proteins. This procedure involves inserting a gene sequence and coding for a *volado* or integrin protein into test cells in culture under conditions where said gene sequence expresses the *volado* or integrin protein in said test cell, adding a test compound to the cell culture or cell homogenate, and measuring the effect of test compound and the activity of the *volado* or integrin proteins. This screening procedure can also be used in organisms such as Drosophila flies.

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# METHOD OF SCREENING FOR NEUROPHARMACEUTICALS USING THE *DROSOPHILA* GENE *VOLADO* AND ITS MUTANTS

The work herein was supported by grants from the United States Government. The United States Government may have certain rights in this invention.

## Field of the Invention

The present invention relates generally to the field of screening for pharmacological agents and drugs which can be used as cognitive enhancers. More specifically, it relates to the field of screening for modulators of integrin function as a screen for cognitive enhancers.

## Background of the Invention

The ability to acquire and process information about the environment (learning) and to store and retrieve this information over time (memory) is fundamental for many organisms. Learning and memory are expressed as modifications of animal behavior (conditioning) which emerge from the function of molecules within neurons, the integrated action of many neurons comprising neural circuits, and from the engagement of multiple circuits.

Two broad phases of memory have been distinguished from behavioral and cellular studies: short-term memory and long-term memory. Short-term memory, which lasts from minutes to hours, is thought to occur through changes in synaptic efficacy produced by rapid and transient biochemical alterations in the relevant neurons. Byrne, J.H. *et al.*, in *Advances in Second Messenger and Phosphoprotein Research* Shenolikar, S. & Nairn, A.C. (eds.) 47-107 (1993); Chetkovich, D.M. *et al.*, *Proc. Natl. Acad. Sci. USA* 88:6467-6471 (1991); Ghirardi, M. *et al.*, *Neuron* 9:479-489 (1992); Davis, R. L., *Physiological Reviews* 76:299-317 (1996);

Hawkins, R.D. *et al.*, *Annual Rev. Neurosci.* 16:625-665 (1993). In contrast, long-term memory, which lasts from days to years, is thought to occur through changes in synaptic efficacy produced by the restructuring of synapses due to altered gene expression. Davis, H.P. & Squire, L.R., *Psychol. Bull.* 96:518-559 (1984); Montarolo, P.G. *et al.*, *Science* 234:1249-1254 (1986); Tully, T. *et al.*, *Cell* 79:35-47 (1994); Schacher, S. *et al.*, *Science* 240:1667-1669 (1988); Bailey, C.H. & Kandel, E.R., *Annual Rev. Physiol.* 55, 397-426 (1993). The formation of long-term memory, but not short-term memory, has therefore been thought to rely upon morphological restructuring of synapses using mechanisms similar to those used for brain development.

In *Drosophila*, the formation of olfactory memories is scripted in cyclic AMP (cAMP) signaling in neurons of the mushroom bodies. Davis, R.L., *Physiological Reviews* 76:299-317 (1996); Davis, R.L., *Neuron* 11:1-14 (1993); Davis, R.L. & Han, K.-A., *Current Biology* 6:146-148 (1996). A significant series of studies linking cAMP signaling, mushroom bodies, and olfactory learning demonstrated that three genes required for normal learning - *dunce* (*dnc*), *rutabaga* (*rut*), and *DCO* (the genes for cAMP phosphodiesterase, adenylyl cyclase, and the catalytic subunit of protein kinase A (PKA), respectively) - are all expressed preferentially in mushroom bodies. Davis, R.L., *Neuron* 11, 1-14 (1993). Moreover, the characterization of two other learning genes of *Drosophila* is consistent with a dominant role for cAMP in modulating the physiology of neurons that mediate behavioral plasticity. The *amnesiac* gene encodes a peptide similar to PACAP (pituitary adenylyl cyclase activating peptide) Feany, M. S. & Quinn, W.G., *Science* 268, 869-873 (1995) and dCREB2 encodes a transcription factor that may mediate cAMP-dependent gene expression. Yin, J.C.P. *et al.*, *Cell* 79:49-58 (1994). A leading hypothesis that has emerged from these studies is that mushroom bodies function as the integration and memory center for olfactory learning by employing the cAMP signaling system. Davis, R.L., *Physiological Reviews* 76:299-317 (1996); Davis, R.L., *Neuron* 11:1-14 (1993).

Mushroom bodies are bilateral clusters of about 2500 neurons situated in the dorsal and posterior cortex of each brain lobe. Davis, R.L. & Han, K.-A., (1996). *Current Biology* 6:146-148 (1996). These cells extend dendrites into a neuropil (calyces) just ventral to the cell bodies where inputs arrive from the antennal lobes and other centers conveying sensory information. The axons of mushroom body cells fasciculate to form the peduncle that projects anteriorly to the anterior of the brain. There it bifurcates, with some processes extending medially to comprise the neuropil region known as the β and γ lobes, and others extending dorsally to comprise the α lobe Strausfeld, N.J., *Atlas of an Insect Brain* (1976). Although the mushroom bodies receive inputs from many sensory modalities through the calyces and lobes and are required for olfactory learning, they are not required for olfaction per se (Heisenberg, M. *et al.*, *Neurogenetics* 2:1-30 (1985); Menzel, R. *et al. The Behavior and Physiology of Bees (L.J. Goodman & R.C. Fisher eds.)* (1991); DeBelle, S.J. & Heisenberg, M., *Science* 263:692-695 (1994).

Despite the coherent evidence pointing to the cAMP signaling system, many different types of molecules must be engaged during learning to effect the overall physiological changes in the relevant neurons. Indeed, an assortment of protein kinases, transcription factors, enzymes involved in neurotransmitter biosynthesis, neuropeptides, and other factors have been suggested to play important roles. Hawkins, R.D. *et al.*, *Annu. Rev. Neurosci.* 16:625-665 (1993); Grant, S.G. & Silva, A.J., *Trends in Neurosciences* 17:71-75 (1994); Alberni, C.M. *et al.*, *Cell* 76:1099-1114 (1994); Mello, C.V. & Clayton, D.F., *J. Neurobiol.* 26:145-161 (1995); Huston, J.P. & Hasenohrl, R.U., *Behav. Brain Res.* 66, 117-127 (1995); Zhuo, M. *et al.*, *Nature* 368:635-639 (1994). The instant invention has isolated a new *Drosophila* memory gene, *Volado* (*Vol*), that encodes a novel α-integrin, a type of cell surface receptor known to dynamically mediate cell adhesion and signal transduction. Hynes, R.O., *Cell* 69:11-25 (1992). "Volado" is

a Chilean colloquialism with no English counterpart, but is loosely translated as "forgetful" or "absent-minded." In Chile, it is often used in reference to professors and scientists.

Lesions in *Vol* have a dominant effect upon short-term memory following olfactory conditioning. Remarkably, conditional expression of *Vol* just before training rescues the memory deficit of *Vol* mutants. This rescue is reversible, supporting a dynamic role for integrins in neuronal and behavioral plasticity. These data indicate that integrin-mediated signaling or synaptic restructuring underlie the formation, stability, or retrieval of short-term memory.

There is a pressing need for the development of new cognitive enhancers. Our abilities to learn, and to forget, are human attributes often taken for granted because they operate in the background of our everyday tasks. Their importance surfaces when these abilities are compromised by head trauma, psychiatric or neurological disease, aging, alcoholism, or from other causes. As many as 5% of school-aged children experience grave difficulties with learning to read and spell. Memory disorders are common among the aged, affecting an estimated 12% of the population over age 65. The personal and financial costs of these disorders are staggering, with the cost of Alzheimer's disease alone at between 80-90 billion dollars each year. Therefore, it is incredibly important to discover effective treatments and cures for the numerous types of cognitive disease.

Despite this obvious need, there are few drugs on the market or in development that have a significant impact upon learning and memory cognitive processes. This is because the mechanisms underlying cognitive processes are complex and the many possible molecular targets have yet to be identified. This perhaps explains the diversity of diseases that affect cognitive processes. It also explains why the pharmaceuticals available are now directed toward enhancing the biological activity of only a handful of molecules. Given the likelihood that perhaps one

hundred different molecules are involved in cognitive processes, any of these, once identified, could potentially be modulated to enhance cognition with appropriate pharmaceuticals.

This discovery deals with the identification of a new molecule involved in cognitive processes. Using a novel methodology to identify genes and molecules involved in Drosophila cognitive processes, molecules of the *volado* family of proteins which are essential for normal cognitive processes formation were discovered. These proteins work by participation in signal transduction cascades and principally, in modulating the activity of tyrosine kinase.

This discovery now makes possible the use of expression systems of *volado* genes to identify pharmaceuticals that increase or decrease their activity.

## SUMMARY OF THE INVENTION

An object of the present invention is a screening system for determining cognitive enhancers using the *Vol* protein in test cells.

An additional object of the present invention is a method for screening for cognitive enhancers using *Vol* or its corresponding mutants.

An additional object of the invention is using integrins or other analogous proteins as substitutes for use of the *Vol* proteins.

Thus, in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention, a method for detecting cognitive enhancers comprising the steps of inserting a gene sequence encoding for *Vol* protein into test cells in culture under conditions where said gene sequence expresses the *Vol* protein in said test cells; adding a test compound to the cell cultures or cell homogenates; and measuring the effect of the test compound on the activity of the *Vol* protein.

In specific embodiments of the present invention, the test cells are selected from a group of invertebrate cells and vertebrate cells. More specifically, they can be mammalian cells selected from the group consisting of human embryonic kidney cells, COS cells or CHO cells, or insect cells selected from Drosophila S2 or Spodoptera SF9 cells with baculovirus vectors.

The specific methods for measuring the activity on *Vol* protein include cell adhesion assays to ligands applied to solid surfaces such as plastic microtiter wells, or to ligands expressed on other cells, in which case the activity can be measured by cell aggregation. Additional specific embodiments use increases in tyrosine phosphorylation within cells expressing the integrin as a measure of integrin activity.

In another specific embodiment of the present invention, the cognitive enhancers are screened by using *volado* mutant Drosophila flies.

Other and further objects, features, and advantages will be apparent from the following description of the presently preferred embodiments of the invention, which are given for the purpose of disclosure, when taken in conjunction with the accompanying drawings.

## Description of the Drawings

Figure 1 shows the *Vol* gene structure, transcripts and mutations. Figure 1A shows an EcoRI (R) restriction map of the locus with the position of the $Vol^1$ enhancer detector element indicated by a triangle. The direction of transcription of the *lacZ* reporter in the enhancer detector element is indicated by the arrow. Two transcription units, *Vol*-long (*Vol*-l) and *Vol*-short (*Vol*-s), were deduced by comparing cDNA sequences with genomic sequences. The first exon of each transcription unit is spliced to a common 2nd exon. Filled boxes represent the open reading frame. The 816 base pair deletion in $Vol^2$ is indicated by the line spanning the first exon of *Vol*-s. Figure 1B shows blots of adult head RNA showing the 4.6 and 4.4 kb transcripts of *Vol*-l and

*Vol*-s, respectively, in Canton-S (cs) and *ry* animals. Figure 1C shows reverse transcriptase (RT)-PCR analyses of total head RNA from rosy (*ry*), $Vol^1$ and $Vol^2$ adults. Each graded bar represents increasing amounts (from left to right) of a single RT reaction added to the subsequent PCR. Both *Vol*-l and *Vol*-s were present in *ry*; however, the expression of *Vol*-l was dramatically reduced in $Vol^1$ and expression of *Vol*-s was undetectable in $Vol^2$. The internal control using PKA primers allowed quantitative comparisons to be made between the various RT-PCR reactions. RNase (+) added prior to the RT reaction abolished all signals.

Figures 2A and 2B show *Vol* preferentially expressed in mushroom bodies. Figure 2A shows a frontal section of a $Vol^1$ adult head stained for β-galactosidase activity. Staining (blue signal) was observed within the mushroom body perikarya (mb). The β-galactosidase encoded by the enhancer detector element carried a nuclear targeting sequence which explains the nuclear localization of the histochemical stain. Figures 2B-2D show frontal sections of Canton-S adults after immuno-staining with an affinity-purified antiserum raised against the carboxy-terminus of *Vol*. Figure 2B shows expression (dark brown signal) observed in the cell bodies (mb) and calyces (c). Figure 2C shows the peduncle (p). Figure 2D shows the α, β, and γ lobes (α, β, γ).

Figure 3 shows memory deficits in *Vol* mutants. Figure 3A shows the decay curve of conditioned odor avoidance for two *Vol* mutants ($Vol^1$ and $Vol^2$) and the control strain (*ry*). N = 8 to 9 for all groups. The mean performance index ± SEM is shown for each genotype at several time points after training. The performance of $Vol^1$ and $Vol^2$ was significantly less than *ry* at all time points. Figure 3B shows the performance of homozygous and heterozygous *Vol* mutants at 3 and 15 minutes after training. N = 8 to 11 for all groups. There were no significant differences between the homozygous mutant strains and the corresponding heterozygous strains at either time point.

Figure 4 shows the lack of neuroanatomical defects in *Vol* mutants. *Ry* and *Vol*$^2$ adult frontal sections are shown at the level of the mushroom body perikarya (mb) and calyces (c) after staining with hematoxylin and eosin (H&E) or with an antibody against the nuclear antigen D-mef2, and at the level of the mushroom body lobes (α, β and γ) after staining with anti-*fasII* or anti-*leonardo* antisera. No differences between the genotypes were observed in either mutant. Slight differences seen here were due to the plane of sectioning. The posterior to anterior arrangement of sections is from top to bottom.

Figure 5 shows rescue of the *Vol* memory defect by conditional expression of *Vol*-s. Figure 5A shows three minute memory without heat shock (NO HS) or 3 h after heat shock (HS 3h) in *ry*, *Vol*$^2$, VS-T2 and VS-T3. Heat shock was for 15 minutes at 37°C. N = 6 for all groups. Rescue of the mutant phenotype was exhibited by both VS-T2 and VS-T3; in addition, VS-T2 exhibited some constitutive rescue. Figure 5B shows RT-PCR analyses of *Vol*-s expression. RT-PCR for *ry*, VS-T2 and VS-T3 without (-) or 30 minutes after HS (+). Figure 5B upper panels show *ry* control: HS had no effect on expression of *Vol*-s or PKA in *ry* animals (compare duplicate lanes 1 and 2 with lane 7, all of which are from PCR reactions containing equivalent amounts of input cDNA). Quantitation using a BetaGen blot analyzer demonstrated that the signals for both *Vol*-s and PKA were linear with the mass of input cDNA (graded bar) amplified by PCR. Figure 5B lower panels show *Vol* transgenics: *Vol*-s RNA was nearly undetectable in both VS-T2 and VS-T3 in the absence of HS (-). Thirty minutes after HS (+) there was a marked induction of the transgene. Lanes 1, 2 and 7 in the upper panels and all lanes in the lower panels are from PCR reactions seeded with equivalent amounts of input cDNA. RNase treatment (+) prior to RT eliminates all signals. Data are representative of three independent experiments. Figure 5C shows *Vol* protein was induced after HS in VS-T3. Immunoblotting was performed on extracts from whole flies without (0h) or 3 and 24 hours after HS. Western blots containing

0.5 fly equivalents per lane were incubated with an affinity-purified antiserum generated against the carboxy-terminus of the *Vol* integrin. This antiserum recognizes both the full-length protein (~135 kDa) as well as the light chain (doublet at ~26 kDa). These data, confirmed by detection with an antiserum generated against the extracellular domain (not shown), are representative of 2 experiments. Figure 5D shows three minute memory without HS (NO HS) or 3 (HS 3h) and 24 h (HS 24h) after HS. N = 6 for all groups. VS-T3 showed a behavioral deficit without heat shock, normal performance with HS 3 h prior to training, but a deficit again when HS was given 24 h prior to training. Figure 5E shows RT-PCR analyses of *Vol*-s RNA expression without (0h) or 0.5 and 21.5 h after HS. Figure 5E upper panels show expression of *Vol*-s was not changed after HS in *ry* animals. Figure 5E lower panels show *Vol*-s RNA was dramatically elevated in VS-T3 0.5 h after HS, and returns to a low level at 21.5 h after HS. As in Figure 5B PKA expression was not changed by HS in either strain. All lanes are from PCR reactions seeded with equivalent amounts of input cDNA. RNase treatment prior to RT eliminates all signals. Data, from a single experiment performed in duplicate, are representative of two independent experiments.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned as well as those inherent therein. The *volado* and integrin proteins along with the DNA and protein sequences, methods, procedures, assays, molecules and specific compounds described herein are presently representative of the preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the claims.

## Detailed Description of the Invention

It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

The term "cognitive processes" as used herein refers to all aspects of intellectual ability, including the elements of problem solving, memory, levels of consciousness, orientation, attention and mental tracking, comprehension, judgment, calculations, reasoning, perception, planning, and constructional ability.

The term "cognitive enhancers" as used herein refers to any agent (for example a compound, composition or drug) that modifies the efficacy of one or more of the elements of the cognitive processes.

The term "mutant" as used herein refers to an alteration of the primary sequence of *volado* thus that it differs from the wild type or naturally occurring sequence. In the nucleic acid sequence, mutant can be any change in the sequence, for example changed base, deletion, or addition which results in an altered protein. In the amino acid sequence, the mutant is a peptide or protein whose sequence is altered from the native sequence.

The term "expression system" as used herein refers to a vector, plasmid or cell that contains all the information necessary to produce a protein from the *volado* gene sequence in *Drosophila* or its corresponding sequence from a mammalian species.

The term "transfection/transfected" as used herein describes the process of directly introducing the DNA into cells including vertebrate cells, invertebrate cells, bacteria and yeast. This includes introducing DNA by transfection of insect cells and mammalian cells.

As used herein, the term "transform/transformation" refers to the result of introducing DNA into a cell where the presence of the DNA genotypically and phenotypically alters a cell in a heritable manner.

The term "reporter" refers to the insertion of a nucleotide sequence downstream from a promoter such that when the promoter is activated the nucleotide sequence is produced in the cell. To be an effective reporter the nucleotide sequence must produce a peptide, protein or other change which can be monitored. For example, it could produce a protein which causes the cells to change color or can be linked to some type of enzyme or antibody reaction in order to detect the presence of the reporter. A skilled artisan readily recognizes that a variety of reporter genes are available for use in the present invention.

The term "*Volado*" as used herein refers to the gene in *Drosophila* which encodes the proteins identified in SEQ ID NOS. 1 and 2. A schematic of the gene and certain mutants is shown in Figure 1.

The terms "$Vol^1$" and "$Vol^2$" as used herein refer to the two *Volado* mutants. A schematic of these two mutants is shown in Figure 1.

One aspect of the present invention is the use of *Volado* proteins to screen for compounds, compositions or drugs to be used as cognitive enhancers. Any agent that increases the biochemical activity of *Volado* proteins in neurons could make cognitive processes more efficient, especially in cases of disease in with the activity of *Volado* proteins is compromised. Clones for the *Drosophila Volado* protein or its mammalian counterparts are used in expression systems for screening new agents that alter the biochemical function of *Volado* proteins.

One skilled in the art readily recognizes that a variety of expression systems can be used. These expression systems can be selected from either invertebrate cells, vertebrate cells, bacteria or yeast.

For example, the expression system can be comprised of mammalian cells. Examples of mammalian cells which are useful in the present invention include mammalian cells in culture, such as human embryonic kidney cells, COS cells, or CHO cells. Examples of invertebrate cells which can be useful in the present invention include insect cells in culture, such as *Drosophila* S2 cells or *Spodoptera* SF9 cells with baculovirus vectors.

Agents that alter the activity of *volado* or integrins are screened for in several different ways. After expression of a *volado* or integrin gene in cultured cells, agents are applied and their ability to modulate *volado* or integrin function is determined by: (1) cell adhesion assays since integrins mediate adhesive functions of cells. These include the binding of expressing cells to ligands deposited on plastic surfaces in microtitre wells or other solid surfaces, or cell aggregation assays in which integrin expressing cells adhere to cells expressing ligands for the integrins, (2) increases in tyrosine phosphorylation, since activation of integrins leads to increased levels of tyrosine kinase. *Drosophila* mutants that have lowered *volado* activity sufficient to cause lethality, poor viability, or learning/memory deficiency are used to screen for agents that alter the *volado* or integrin activity. Agents with this property rescue the viability defects and/or the defects in learning/memory *Drosophila* mutants that have a lowered *volado* activity sufficient to cause lethality, poor viability, or learning/memory deficiency are used to screen for agents that alter the *volado* or integrin activity. Agents with this property rescue the viability defects and/or the defects in learning/memory.

The predicted amino acid sequence of *Vol*-l and *Vol*-s are indicated as SEQ ID NO. 1 and SEQ ID NO. 2 respectively. The complete amino acid sequence for *Vol*-l is contained in SEQ ID NO. 1. The first 63 amino acids of *Vol*-s are listed as SEQ ID NO. 2.

**Example 1**
**The *Vol* Locus Encodes a Novel α-integrin**

Approximately 6000 enhancer detector lines were constructed and screened for preferential expression of the *lacZ* reporter in brain structures as is well known in the art. (*See e.g.*, Han, P.-L. *et al.*, *J. Neurobiol.* 31:88-102 (1996)). About 100 lines with preferential mushroom body expression were isolated, including insertions at the *dnc*, *rut*, *DCO* and *leonardo* (*leo*) genes. Line 1116 ($Vol^1$) from this screen also expressed *lacZ* in mushroom bodies; the enhancer detector element in this line was mapped to cytological position 51E.

The region flanking the enhancer detector element was isolated along with wild type genomic and cDNA clones for the locus. The locus is organized into two transcription units, *Vol*-long (*Vol*-l) and *Vol*-short (*Vol*-s), which encode RNAs of 4.6 and 4.4 kb, respectively (FIG. 1). The *Vol*-l RNA is expressed selectively in heads, whereas *Vol*-s is expressed in both head and body tissues. Mapping experiments showed that the $Vol^1$ enhancer detector element resides within the first intron of *Vol*-l and within the 5' flanking region of *Vol*-s (FIG. 1A). Imprecise excision of the element led to the isolation of $Vol^2$, an allele with an 816 nucleotide deletion of genomic sequence that removes the first exon of *Vol*-s (FIG. 1A). Reverse-transcriptase (RT)-PCR analyses of head RNA revealed that expression of the *Vol*-l transcript was greatly reduced in $Vol^1$, while the *Vol*-s transcript was unaffected (FIG. 1C). Conversely, the $Vol^2$ lesion eliminated the *Vol*-s transcript without discernible changes in *Vol*-l (FIG. 1C). Neither allele affected the expression of PKA, the internal control in these experiments (FIG. 1C). The effects of the alleles on expression of the two transcripts, as confirmed by RNA blotting experiments are consistent with the nature of the physical lesions at the gene (FIG. 1A). Thus, the $Vol^1$ and $Vol^2$ alleles disrupt *Vol*-l and *Vol*-s expression, respectively.

The cDNAs for *Vol*-l and *Vol*-s predict novel α-integrins of 1115 amino acids differing only in the first 63 amino acids (Fig. 1A, SEQ ID NO. 1, SEQ ID NO. 2). The *Vol* proteins contain many hallmarks of other α-integrins. The *Vol* proteins are 23-28% identical in amino acid sequence with known α-integrins and contain a single transmembrane domain near the carboxy-terminus. The proteins begin with 24 residues of a hydrophobic, putative signal peptide, have 11 potential glycosylation sites [NXT(S)] in the extracellular region, and have three repeats in the extracellular region that match the consensus DX(D/N)X(D/N)GXXD, which is well known in the art to be a domain found in proteins that bind divalent cations. (*See e.g.*,Kretsinger, R.H., *CRC Critical Reviews in Biochemistry* 8:119-74 (1980)). Moreover, the *Vol* sequence has a cleavage recognition site (RKRR) in the extracellular domain, a site required for signal transduction by some α-integrins. After cleavage at these sites, the amino-terminal and carboxy-terminal integrin fragments are held together by disulfide bonds. Furthermore, the cytoplasmic domain of *Vol* contains the consensus sequence, KXFF[K/R]R, which is known in the art to bind calreticulin (*See e.g.*, Dedhar, S., *Trends in Biochem. Sci.* 19:269-307 (1994)) and regulate integrin affinity for ligand.

**Example 2**
**Expression of *Vol* in Mushroom Bodies**

The $Vol^1$ mutant preferentially expressed the *lacZ* reporter in the nuclei of mushroom body neurons (FIG. 2A). To determine if the enhancer detector reflected authentic *Vol* protein expression, immunohistochemical analyses with an antiserum made against the carboxy-terminus of the protein were performed. The *Vol* antigen was found to be concentrated in the mushroom body perikarya and calyces (FIG. 2B), peduncles (FIG. 2C), and α, β, and γ lobes (FIG. 2D). The calyces, peduncles, and lobes contain the mushroom body dendrites, axons, and axon terminals,

respectively. The distribution of the antigen was not noticeably altered in either the *Vol*$^1$ or *Vol*$^2$ mutants (data not shown), suggesting that both *Vol*-l and *Vol*-s isoforms are globally co-expressed in the mushroom bodies. Enriched expression was also observed in the ellipsoid body (not shown), a region of the central complex thought to be involved in the coordination of motor behaviors. The distribution of *Vol* in the mushroom body calyces and lobes - regions in which mushroom body neurons form synapses with other neurons suggests that the *Vol* integrins could regulate synapse function.

**Example 3**
**Mutations in *Vol* Produce a Memory Deficit**

The expression pattern of *Vol*, coupled with preliminary behavioral experiments, suggested that this gene is important for olfactory memory. To test this hypothesis, Vol mutants were assayed for aversive olfactory classical conditioning. Populations of animals were administered electric shock (unconditioned stimulus, US) in the presence of one odor, the conditioned stimulus (CS+), and were subsequently presented a second odor (CS-) without shock. To evaluate discriminative avoidance behavior, the trained animals were allowed to distribute between converging CS+ and CS- odors carried in air currents within a T-maze.

Animals homozygous for the *Vol*$^1$ insertion or the *Vol*$^2$ deletion performed poorly relative to *ry* at all time points after training (FIG. 3A; genotype, P = .0001; retention interval, P = .0001; genotype x retention interval, NS). The effects of these mutations on memory were indistinguishable, suggesting that the two integrin isoforms are functionally redundant. It had been shown previously that neither the enhancer detector itself, nor the expression of *lacZ* in mushroom bodies per se, have any significant effect upon performance. The performance deficits in *Vol* mutants were present at the earliest testable time point after training (3 minutes), indicating

that the formation, stability, or retrieval of short-term memory is dependent upon integrin function.

To further examine the effects of the *Vol* alleles on early memory and to investigate their recessive or dominant nature, the performance of animals heterozygous or homozygous for the two lesions of the gene was trained and tested. The $Vol^1$ and $Vol^2$ animals exhibited memory deficits at both 3 and 15 minutes after training (FIG. 3B; 3 minutes, P = .0001; 15 minutes, P = .0001), confirming the results in Figure 3A. The performance index (PI) of the $Vol^1$/+ and $Vol^2$/+ heterozygous animals was similarly reduced relative to *ry*, but was not significantly different from the corresponding homozygous mutants (FIG. 3B; $Vol^1$ vs. *ry* at 3 minutes, P = .0001; at 15 minutes, P = .0001; $Vol^2$ vs. *ry* at 3 minutes, P = .00015; at 15 minutes, P = .0001; $Vol^1$ vs. $Vol^1$/+ at 3 minutes, NS; at 15 minutes, NS; $Vol^2$ vs. $Vol^2$/+ at 3 minutes, NS; at 15 minutes, NS). Trans-heterozygous animals, $Vol^1/Vol^2$, also exhibited a PI equivalent to $Vol^1$/+ or $Vol^2$/+. Thus, as with *dnc*, *rut*, *turnip*, *radish*, and *cabbage*, mutations in *Vol* have a dominant effect on memory. The dominant effect is particularly noteworthy for *Vol* alleles, since three out of the four transcription units were preserved in animals heterozygous for *Vol*-l or *Vol*-s lesions. These data support the existence of a threshold requirement for *Vol* expression in the processes underlying memory, making them acutely sensitive to decreased expression of this gene.

**Example 4**
**Evaluation of *Vol* Sensorimotor Processes**

To eliminate the possibility that the poor performance of *Vol* mutants was due to defects in sensorimotor processes, their ability to sense and avoid electrical shock pulses and the odors used for conditioning was tested. The avoidance behavior of *Vol* mutants and control animals to electrified grids and odors used for conditioning at multiple strengths of these stimuli was

indistinguishable. For example, the avoidance indices to 0.8 ml octanol were 63±4, 68±4, and 65±5 for *ry*, *Vol*$^1$ and *Vol*$^2$, respectively. The morphology of the brain was explored with a particular emphasis on mushroom bodies to determine whether the poor performance was attributable to defects in brain structure. Serial paraffin sections of control and mutant brains failed to reveal any discernible differences in morphology when stained with Hematoxylin and Eosin (H&E); an antibody against the nuclear antigen D-mef2, which reveals a subset of mushroom body cell nuclei; an antibody against the *leo* gene product, which delineates the mushroom body calyces, cell bodies, peduncles, and lobes, or an antibody against fasII, which reveals a subset of the mushroom body lobes (FIG. 4). Therefore, neither sensorimotor or gross neuroanatomical defects can account for the memory deficit of *Vol* mutants.

**Example 5**
**Conditional Rescue of the *Vol* Memory Deficit**

Direct evidence for a role of the integrin in physiological processes underlying memory is obtained through the conditional expression of a *Vol* transgene. Four transgenic lines were generated that harbored the *Vol*-s cDNA under the control of the hsp70 promoter in the *Vol*$^2$ background (*Vol*-s mutant). Animals were heat-shocked for 15 minutes at 37°C, rested for 3 hours to allow for recovery and expression of the transgene, and subsequently trained and tested for 3 minute memory. Two of the transgenic lines failed to show any evidence of heat-dependent rescue in pilot experiments, presumably due to genomic position effects, and were not analyzed further. Two other lines, VS-T2 and VS-T3, were analyzed extensively for olfactory memory.

Normal olfactory memory of *ry* control animals and the residual memory in Vol$^2$ mutants was unaffected by heat shock (FIG. 5A; for *ry*, NO HS vs. HS 3h, NS; for Vol$^2$, NO HS vs. HS 3h, NS). In the absence of heat shock, VS-T3 transgenic animals exhibited mutant levels of

performance, but VS-T2 transgenic animals showed partial rescue of memory, possibly due to elevated basal expression of the transgene in mushroom bodies (FIG. 5A; NO HS, VS-T3 vs. *ry*, P = .0001; VS-T3 vs. $Vol^2$, NS; VS-T2 vs. *ry*, P = .0008; VS-T2 vs. $Vol^2$, P = .0003). However, the 3 minute memory of VS-T2 and VS-T3 animals when tested 3 hours after heat shock, was significantly improved over that after no heat shock and was indistinguishable from the *ry* control (FIG. 5A; HS vs. NO HS, VS-T2, P = .0045; VS-T3, P = .0005; with HS, *ry* vs. VS-T2, NS; *ry* vs. VS-T3, NS). Therefore, conditional expression of *Vol*-s just before behavioral training was sufficient to fully rescue the mutant phenotype. This rescue cannot be attributed to altered sensorimotor abilities, since avoidance behavior to electric shock and odors by the control and transgenic animals was indistinguishable, with or without heat shock. These data provide compelling evidence that the defective α-integrin expression in *Vol* mutants is responsible for the memory deficits, and that the *Vol* integrin participates in the physiological processes underlying memory.

To determine whether the behavioral rescue was paralleled by the induction of the *Vol* transgene, *Vol* RNA and protein levels were assayed before and after heat shock. As assayed by RT-PCR, heat shock had no effect on the quantity of *Vol*-s RNA in *ry* control animals (FIG. 5B), but produced a ~100-fold and ~1000-fold increase in the level of *Vol*-s RNA in the VS-T2 and VS-T3 transgenic lines, respectively (FIG. 5B). The level of PKA RNA served as an internal control and was unaffected by *Vol* mutation (FIG. 1B), *Vol* transgene expression, or heat shock (Fig. 5B). Western blotting was used to measure *Vol* protein using an affinity-purified antiserum raised against the carboxy-terminus of *Vol* that recognized the intact *Vol* protein (Mr ≈ 125 kDa), as well as the carboxy terminal cleavage fragment produced by proteolysis (Mr ≈ 21 kDa). This antiserum identified a band (sometimes a doublet) of 26 kDa in *ry* that was not found in $Vol^2$ mutants or in non-heat shocked transgenics animals (FIG. 5C). This band represents the

carboxy-terminal cleavage fragment. The full-length protein was not detected in *ry* extracts, presumably due to reduction of the disulfide bond that links the heavy and light chains. In contrast to the *ry* control, a large increase in the expression of both the *Vol* full-length protein and light chain was found in VS-T3 extracts obtained three hours after heat shock (FIG. 5C). Detection of the intact molecule suggests that the protease is limiting after over-expression of *Vol*. Induction of *Vol* protein was also observed in VS-T2. Thus, there was a marked elevation of the *Vol* α-integrin in the VS-T2 and VS-T3 transgenics 3 hours after heat shock. These RNA and protein analyses demonstrated that *Vol* was conditionally expressed at the time of behavioral assay, confirming that replacement of the *Vol* integrin in adulthood rescued the memory deficit.

Despite the arguments presented above for a physiological role for *Vol*, it seemed plausible that the α-integrin might be required for a final step in synapse formation that occurs normally during development, and that the induced expression of the integrin during adulthood simply allows completion of this terminal step. In other words, the presence of the integrin might be essential for synapse formation but not for synapse stability. If so, the induction of *Vol* expression might cause a long-lasting or permanent rescue of memory. If, on the other hand, *Vol* participates in a non-developmental, acute aspect of neuronal function, the rescue of memory produced by induction of the *Vol* transgene would be expected to be transient and reversible, persisting only as long as adequate levels of the *Vol* integrin are present.

To distinguish between these possibilities, whether induction of *Vol* produced a permanent or a reversible restoration of memory was explored. As before (FIG. 5A), heat shock treatment 3 hours prior to training and testing dramatically improved the performance of VS-T3 animals (FIG. 5D; for VS-T3, NO HS vs. HS 3h, $P = .0001$). This rescue was completely reversible. The memory in heat-shocked VS-T3 transgenic animals returned to mutant levels when the animals

were trained and tested 24 hours after heat shock (Fig. 5D; for VS-T3, no HS vs. HS 24h, NS; HS 3h vs. HS 24h, P ..0001; for HS 24h, *ry* vs. VS-T3, P = .0001; $Vol^2$ vs. VS-T3, NS).

*Vol* RNA and protein expression in the transgenic animals, which reflect abundance in all cells, were markedly elevated at early time points after heat shock (0.5 and 3 hours, respectively), and decreased to low levels at late time points (21.5 and 24 hours, respectively) (FIGS. 5C, 5E). Thus, the induction and ensuing decline of *Vol* expression correlated well with the behavioral rescue and subsequent return to a state of memory impairment. The temporal parallels in RNA level, protein expression, and memory argue strongly that *Vol* mediates a physiological process that is critical to memory formation, stability, or retrieval.

Collectively, these results support three important points. First, reduced expression of the *Vol* integrin produces an impairment in memory without altering sensorimotor abilities or neuroanatomy. Second, this phenotype is rescued by the expression of the integrin just before training in the adult animal, demonstrating an adult role for this adhesion molecule. Third, the reversibility of the memory rescue indicates that the *Vol* integrin mediates a dynamic process underlying memory.

## Example 6
## Integrins, Synaptic Plasticity, Mushroom Bodies and Memory

The results of the identification, isolation, and characterization of *Vol* properties of *Vol* similar to those seen in studies of four other learning genes with similar expression patterns: *dnc*, *rut*, *DCO* and *leo*. (For examples of other learning genes *see e.g.*, Davis, R.L., *Neuron* 11:1-14 (1993); Skoulakis, E.M.C. & Davis, R.L., *Neuron* 17:931-44 (1996). Nighorn, A. *et al.*, *Neuron* 6:455-467 (1991); Han, P.-L. *et al.*, *Neuron* 9:619-627 (1992); Skoulakis, E. *et al.*, *Neuron* 11:197-208 (1993)). The discovery of another memory mutant in which the underlying gene is

expressed preferentially in mushroom bodies reinforces the conclusion that these cells play a crucial role in olfactory learning and memory. The mushroom bodies may serve as centers for the reception and integration of many different forms of sensory information, including information about odors and electric shock presented during olfactory classical conditioning. The converging sensory information is thought to alter the physiology of mushroom body cells to encode memory, employing the cAMP signalling system as well as other types of molecules. The results with *Vol* demonstrate that integrins are included in the family of molecules required for memory formation.

Integrins have diverse biological roles in apoptosis, cell cycle regulation, cell migration, blood clotting and leukocyte function. They function as αβ heterodimers, mediating adhesive interactions of cells with the extracellular matrix or with counter-receptors displayed by other cells. Most interestingly, they dynamically transduce information across cell membranes bi-directionally. Ligand binding to integrins induces a variety of signalling events within cells, and agonist activation of classical signal transduction pathways can alter the affinity of integrins for their ligands within a time-frame of a few minutes.

The dynamic adhesion role for integrins offers a hypothesis for how the *Vol* integrin, and integrins in general, underlie alterations in synaptic plasticity and behavior. It is envisioned that release of a modulatory neurotransmitter upon a mushroom body neuron might mobilize the intracellular events leading to an altered binding of integrins displayed at another synapse made by that cell. For example, protein kinase C or ras activation is known to activate integrin binding. This could produce a rapid (within minutes) alteration in the structure and efficacy of that synapse. The modulation of integrin affinity for ligands might also underlie the construction or pruning of existing synapses, or the activation of silent synapses during learning or memory encoding. Thus, the formation of short-term memory may employ synaptic rearrangements like long-term memory, but through an integrin-dependent, and protein synthesis-independent

mechanism. Alternatively, it is possible that integrins modulate neuronal function through ligand binding followed by activation of intracellular signalling events. For example, integrins are known to stimulate a number of signal transduction pathways in many types of cells, including $Ca^{2+}$ mobilization, tyrosine kinase activation, and induction of protein kinase C. Integrin-dependent stimulation of these pathways in the relevant neurons may be fundamental to learning and memory.

The results demonstrating a role for integrins in behavioral plasticity mesh well with studies showing integrin-dependent modulation of synaptic plasticity. Notably, peptide inhibitors of integrin binding have no effect upon the formation of long-term potentiation, but block the maintenance of this form of synaptic plasticity. In addition, the enhancement of neurotransmitter release from motor nerve terminals due to muscle stretch is blocked by the peptide inhibitors. Psychological studies coupled with these behavioral studies, support a model in which integrins mediate dynamic processes at synapses underlying memory formation or stability.

## EXAMPLE 7
## Cloning, Mutagenesis and Transgenic Animals

Genomic sequences flanking the *Vol*$^1$ insertion were isolated by plasmid rescue. Wild-type genomic clones were isolated from a Canton-S library made in lambda DASHII; cDNA clones were isolated from libraries prepared from *Drosophila* head RNA. The 4.6 kb *Vol*-l RNA sequence is represented by a cDNA of ~4600 residues. The 4.4 kb *Vol*-s RNA is represented by a 3366 bp cDNA.

The *Vol*$^2$ excision was isolated after dysgenesis. Flies carrying the *Vol*$^1$ enhancer detector element were crossed to Xcs; CyO/2cs; *ry* Sb P[ry+,D2-3,99B]/TM6,Tb. ("cs" denotes chromosomes derived from a wildtype Canton-S stock.) Dysgenic progeny carrying CyO were

crossed to Xcs; CyO/leo$^{1375}$; $ry^{506}$-iso animals. CyO; $ry^{506}$-iso progeny were selected for stocks. $ry^{506}$-iso is an isogenic $ry^{506}$ chromosome. Excision derivatives were characterized by Southern blotting, extensive PCR analyses, and sequencing of PCR products that cross deletion break points.

Due to the nonspecific behavioral effects of mini-white vectors, a new P-factor vector (pCy-20-dbhsp) for driving genes behind the hsp70 promoter was constructed with *ry+* as the selectable marker. This vector, containing a MluI-KpnI fragment of the *Vol*-s cDNA was injected into *Vol*$^2$ embryos. Chromosomal localization of the transgenes and the generation of homozygotes for the transgenes were performed by standard crosses. The presence of the *Vol*$^2$ allele in the transgenic animals was confirmed by PCR analyses of genomic DNA. The *Vol* transgene resides on the X and 2nd chromosome, respectively, in VS-T2 and VS-T3.

Flies were collected in clean food vials, transferred to pre-warmed food vials, and immersed in a 37°C water bath for 15 minutes. Following heat-shock, flies were transferred to room-temperature food vials and stored until testing.

## EXAMPLE 8
## RNA Blots and RT-PCR Analyses

For RNA blots, polyA+ RNA was isolated after tissue homogenization in guanidinium-isothiocyanate, banding in CsCl gradients, and by batch adsorption to oligo-(dT) cellulose. Ten µg polyA+ RNA was fractionated per lane by formaldehyde-agarose gel electrophoresis. For RT-PCR experiments, total RNA from heads or whole flies was extracted using Trizol (Gibco-BRL) according to the manufacturer's instructions. Each RT reaction contained 3 µg total RNA, 500 ng oligo-(dT), and 200 U SuperScript II (Gibco-BRL) in a total volume of 20 µl. The reactions were incubated at 42°C for 50 minutes and digested with 10 U

*Sau*3AI and 10 U *Aci*I at 37°C for 3 hours. RNase A treatments (10 µg) prior to RT reactions were for 1 hour at 37°C. Aliquots of 0.2-5.0% of the RT reactions were amplified using PCR for 20 cycles. For amplification of *Vol* first-strand cDNAs, an antisense primer that anneals to the common 2nd exon of *Vol* (857 nucleotides 3' of translation start site) was used in combination with sense primers specific for the first exon of either *Vol*-l or *Vol*-s (84 and 118 nucleotides 5' to translation start site, respectively). For amplification of PKA, primers that anneal to the 2nd exon of *DCO* were used. PCR products (942, 975 and 356 bp for *Vol*-l, *Vol*-s and PKA, respectively) were electrophoresed in agarose gels, blotted, and hybridized to 32P-labelled probes.

## EXAMPLE 9
## Histology, Generation of Antisera, and Immunoblotting

β-galactosidase staining and H&E staining was performed as is known in the art. (*See e.g.*, Han, P.-L. *et al.*, *J. Neurobiol.* 31:88-102 (1996); Skoulakis, E.M.C. & Davis, R.L., *Neuron* 17:931-44 (1996); Han, P.-L. *et al.*, *Neuron* 9:619-627 (1992); Skoulakis, E.M. *et al.*, *Neuron* 11:197-208 (1993); Han, K.-A. *et al.*, *Neuron* 16:1127-35 (1996)).

For generation of antisera, rabbits were injected with a purified GST-*Vol* fusion protein containing either *Vol* amino acid sequence 1087-1115 (carboxy-terminus) or 358-496 (extracellular domain). For immunohistochemistry using anti-*Vol* antisera and the anti-fasII monoclonal antibody 1D4, adult heads were fixed in 4% paraformaldehyde at 4°C for 2 hours and incubated in 25% sucrose in Ringer's solution at 4°C overnight. Ten mm serial cryosections were incubated with affinity-purified anti-Vol or anti-fasII antibody at 4°C overnight. For anti-D-mef2 and anti-leonardo staining, adult heads were fixed in Carnoy's for 4 hours, embedded in paraffin, sectioned and incubated with the appropriate antiserum overnight at 23°C. In all cases, the antigen/antibody complexes were visualized using the Elite Vectastain ABC kit (Vector

Laboratories). For immunoblotting, protein extracts were prepared by homogenizing whole flies in 2X Laemli's sample buffer containing 1% β-mercaptoethanol at 75°C for 30 minutes. Fly extracts (0.5 fly equivalents per lane) were electrophoresed on SDS-polyacrylamide gels and blotted onto PVDF membranes (Millipore). Blots were incubated with affinity-purified anti-*Vol* sera overnight at 4°C, HRP-conjugated goat-anti-rabbit IgG (Jackson Laboratories) for 1 hour at 23°C, and visualized with SuperSignal Chemiluminescent substrate (Pierce).

## EXAMPLE 10
## Behavioral Analyses

The differential olfactory conditioning paradigm pairing the presentation of one odor with aversive shock and a second odor with the absence of shock, was used to assess learning and memory performance. Training and testing were performed blind to strain under dim red light at 23-25°C and 63-68% relative humidity using procedures well known in the art. (*See e.g.*, Skoulakis, E.M.C. & Davis, R.L., *Neuron* 17:931-944 (1996)). In each group, a performance index (PI) was calculated as the fraction of flies that avoided the CS+ minus the fraction of flies that avoided the CS-, and multiplied by 100. In practice, PI scores ranged from 0 (naive behavior) to 100 (perfect performance). Because the minimum possible time between training and testing is 3 minutes (due to handling and recovery of flies after transfer), 3 minute memory reflects the earliest testable time point. To test longer-term memory, the flies were returned as a group to their collection vials for the appropriate retention interval and then tested as above. Odor avoidance was calculated as the fraction of flies that avoided the odor in one arm minus the fraction of flies that avoided fresh air (and multiplied by 100) provided in the control arm. Electroshock avoidance was calculated similarly.

## EXAMPLE 11
### Statistics

Statistical analyses were performed with Statview 2.0 (Abacus Concepts, Berkeley, CA). Overall ANOVAs were followed by planned comparisons contrasting the relevant groups. Error rate due to multiple comparisons was controlled by dividing the alpha level by the number of comparisons being performed on a given set of data.

## REFERENCES

All patents and publications mentioned in this specification are indicative of the level of those skilled in the art to which the invention pertains. All patents, publications herein are incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1115 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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1               5                  10                  15

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            20                  25                  30

Arg Val Ile Asn Ser Pro Lys His Leu Lys Phe His Ile Asn Gln Thr
        35                  40                  45

Arg Ser Ser Tyr Phe Gly Tyr Thr Leu Val Ile Arg Gln Thr Ser Ile
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Ile Val Gly Ala Pro Arg Ala Gln Ser Thr Leu Glu Ser Gln Arg Thr
65                  70                  75                  80

Ile Asn Glu Thr Gly Ala Ile Tyr Arg Cys Ser Leu Thr Asn Gly Val
                85                  90                  95

Cys Ser Pro Tyr Val Leu Asp Ser Arg Gly Asn Val Asp Ala Pro Tyr
            100                 105                 110

Ser Glu Tyr Thr Phe Asp Ser Glu Arg Lys Asp Phe Gln Trp Leu Gly
        115                 120                 125

Gly Ser Met Asp Gly Gly Thr Lys Asp Thr Asp Lys Leu Leu Val Cys
    130                 135                 140

Ala Pro Arg Phe Tyr Ala Pro Ser Ser Arg Asp Asn His Leu His Gly
145                 150                 155                 160

Val Cys Tyr Trp Val Asn Asn Thr Val Ala Ser Thr Pro Gln His Val
                165                 170                 175

Thr Arg Ile Ser Pro Leu Arg Leu Lys Ser Glu Gln Val Lys Glu Glu
            180                 185                 190

Asp Asn Gly Asn Lys Ala Ser Phe Phe Tyr Ile Met Gly Glu Leu Gly
        195                 200                 205

Leu Ser Ala His Val Ala Asp Asp Asn Thr Lys Phe Leu Ile Gly Ala
    210                 215                 220

Pro Gly Ile Asn Thr Trp Arg Gly Ser Val Ile Leu Tyr Arg Gln Val
225                 230                 235                 240

Asp Pro Val Asp Asn Pro Thr Ala Ser Arg Arg Asp Thr Ser Lys Ala
                245                 250                 255

Leu Arg Arg Thr Tyr Arg Asp Val Asp Ser Asn Asp Tyr Thr Pro Glu
            260                 265                 270
```

His Tyr Ala Pro Glu Ile Pro Thr Pro Gly Leu Trp Gly Gln Glu Glu
275 280 285

Asp Ser Tyr Phe Gly Tyr Ala Val Ser Ser Gly Phe Phe Asp Ser Ser
290 295 300

Asn Pro Thr Lys Leu Leu Tyr Val Ala Thr Ala Pro Gln Ala Asn Lys
305 310 315 320

Gln Ser Gly Glu Ala Tyr Ile Phe Asp Val Arg Gly Lys Ser Ile His
325 330 335

Lys Tyr His Val Phe Arg Gly Glu Gln Phe Gly Glu Tyr Phe Gly Tyr
340 345 350

Ser Val Leu Ala Glu Asp Leu Asn Gly Asp Gly Lys Thr Asp Val Ile
355 360 365

Val Ser Ala Pro Gln His Ala Leu Glu Asp Ser His Asp Asn Gly Ala
370 375 380

Ile Tyr Val Phe Ile Asn Lys Gly Phe Phe Asn Phe Glu Arg Gln Ile
385 390 395 400

Leu Arg Ser Pro Val Glu Thr Met Ala Arg Phe Gly Thr Ala Leu Ser
405 410 415

Arg Leu Gly Asp Ile Asn His Asp Gly Tyr Asn Asp Val Ala Val Gly
420 425 430

Ala Pro Phe Ala Gly Asn Gly Thr Val Phe Ile Tyr Leu Gly Ser Glu
435 440 445

Asn Gly Leu Arg Asp Gln Pro Ser Gln Arg Leu Asp Ala Pro Ser Gln
450 455 460

Gln Pro Ser Lys Tyr Gly Ser His Met Phe Gly His Gly Leu Ser Arg
465 470 475 480

Gly Ser Asp Ile Asp Gly Asn Gly Phe Asn Asp Phe Ala Ile Gly Ala
485 490 495

Pro Asn Ala Glu Ala Val Tyr Leu Tyr Arg Ala Tyr Pro Val Val Lys
500 505 510

Val His Ala Thr Val Lys Ser Glu Ser Arg Glu Ile Lys Pro Glu Gln
515 520 525

Glu Lys Val Lys Ile Thr Ala Cys Tyr Arg Leu Ser Thr Thr Ser Thr
530 535 540

Asp Lys Leu Val Gln Glu Gln Glu Leu Ala Ile Arg Ile Ala Met Asp
545 550 555 560

Lys Gln Leu Lys Arg Val Lys Phe Thr Gln Thr Gln Thr Asn Glu Ile
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Ser Phe Lys Val Asn Ala Asn Phe Gly Glu Gln Cys Arg Asp Phe Glu
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Thr Gln Val Arg Tyr Ser Glu Lys Asp Ile Phe Thr Pro Ile Asp Leu
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610 615 620

Cys Glu Thr Cys Ala Val Val Asp Pro Thr Glu Pro Lys Val Ser Thr
625 630 635 640

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645 650 655

Asp Leu Gln Leu Arg Ser Lys Asn Val Ser Pro Thr Tyr Ile Leu Gly
660 665 670

Ser Ala Asp Thr Leu Arg Leu Asn Tyr Glu Ile Thr Asn Ile Gly Glu
675 680 685

Thr Ala Tyr Leu Pro Gln Phe Asn Val Thr Ser Thr Ser Arg Leu Ala
690 695 700

Phe Ala Gln Val Pro Gly Asn Cys Lys Val Val Asp Ala Val Met Val
705 710 715 720

Cys Asp Leu Asn Arg Gly Arg Pro Leu Ala Lys Gly Asp Thr Asp Ser
725 730 735

Val Thr Ile Ser Phe Asp Val Ser Gln Leu Ser Gly Gln Ser Leu Ile
740 745 750

Ser His Ala Glu Val Phe Ser Thr Gly Tyr Glu Gln Asn Pro Thr Asp
755 760 765

Asn Arg Gln Thr Asn Val Ile Gly Leu Lys Glu Phe Thr Glu Ile Asp
770 775 780

Ala Ser Gly Gly Gln Thr Asn Arg Gln Ile Asp Leu Glu His Tyr Ser
785 790 795 800

Asn Ser Ala Glu Ile Val Asn Asn Tyr Glu Ile Lys Ser Asn Gly Pro
805 810 815

Ser Val Ile Glu Gln Leu Thr Val Ser Phe Tyr Ile Pro Ile Ala Tyr
820 825 830

Lys Val Ala Gly Ser Thr Ala Ile Ile Pro Ile Ile Asn Val Thr Ser
835 840 845

Leu Lys Met Gln Ala Ser Tyr Asp Ser Gln Leu Leu Ser Ile Asp Leu
850 855 860

Tyr Asp Gln Asn Asn Thr Met Leu Val Val Asp Pro Val Glu Val Thr
865 870 875 880

Thr Thr Leu Ser Gly Gly Leu Glu Arg Thr Val Ile Thr Gln Asn Arg
885 890 895

Gln Ser Tyr Asp Ile His Thr Ser Gly His Val His Gln Thr Met Glu
900 905 910

Val Leu Asp Thr Ser Met Val Ala Thr Ala Ser Met Ser Arg Lys Arg
915 920 925

Arg Asp Leu Lys Ala Leu Thr Ala Asn Arg Glu Gln Tyr Ala Arg Ile
930 935 940

Ser Asn Val Lys Ala His Asp Leu Leu Ser Asp Asp Phe Lys Gly Lys
945 950 955 960

Leu Pro Val Asn Arg Thr Ile Val Phe Asn Cys Arg Asp Pro Glu Met
965 970 975

Thr Ile Cys Val Arg Ala Glu Met Arg Val His Phe Arg Pro Glu Lys
980 985 990

Ser Ile Asn Leu Asn Met Arg Tyr Ser Val Asp Leu Asn Glu Val Asn
995 1000 1005

Ala Ile Leu Val Asp Pro Trp Glu Tyr Phe Val Ile Leu Thr Asp Leu
1010 1015 1020

Lys Leu Gln Lys Lys Gly Asp Pro Thr Ser Thr Ser Phe Ser Ile Asn
1025 1030 1035 1040

Arg Arg Ile Glu Pro Asn Ile Ile Ser Lys His Gln Glu Thr Gly Leu
1045 1050 1055

Pro Ile Trp Ile Ile Ile Val Ser Val Ile Gly Gly Leu Leu Leu Leu
1060 1065 1070

Ser Ala Ile Ser Tyr Leu Leu Tyr Lys Phe Gly Phe Phe Asn Arg Thr
1075 1080 1085

Lys Lys Asp Glu Leu Asp Arg Leu Val Gln Gln Asn Pro Val Glu Pro
1090 1095 1100

Glu Ala Glu Asn Leu Asn Ser Gly Gly Asn Asn
1105 1110 1115

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Val Gly Gln Asp Arg Asp Phe Trp Ala Leu Leu Val Leu Gly Leu
1 5 10 15

Trp Cys Leu Ser Ser His Cys Asn Ala Phe Asn Leu Ser Pro Leu Pro
20 25 30

Asn Arg Gln Ile Leu Asp Pro Gln Phe Ala Thr Asn Leu Pro Lys Val
35 40 45

Arg Ala Ser Tyr Phe Gly Phe Thr Met Ser Leu Arg Pro Asn Gly
50 55 60

We claim:

1. A method for detecting a test compound for cognitive enhancer activity, comprising the steps of:

inserting a gene sequence encoding for a protein involved in cognitive processes into test cells in culture under conditions where said gene sequence expresses the protein involved in cognitive processes in said test cell;

adding the test compound to the cell culture or cell homogenates; and

measuring the effect of the test compound on the activity of the protein involved in cognitive processes.

2. The method of claim 1, wherein the protein is a *Volado* protein.

3. The method of claim 1, wherein the protein is an integrin protein.

4. The method of claim 1, 2, or 3 wherein the test cells are selected from the group consisting of invertebrate cells and vertebrate cells..

5. The method of claim 1, 2, or 3 wherein the test cells are mammalian cells.

6. The method of claim 5, wherein the mammalian cells are selected from the group consisting of human embryonic kidney cells, COS cells and CHO cells.

7. The method of claim 1, 2, or 3 wherein the cells are insect cells.

8. The method of claim 7, wherein the cells are selected from the group consisting of *Drosophila* S2 cells and *Spodoptera* SF9 cells with baculovirus vectors.

9. The method of claim 1, 2, or 3 wherein the measuring step includes determining the degree of cell adhesion to ligands applied to solid surfaces.

10. The method of claim 9 wherein the solid surface is a plastic microtiter well.

11. The method of claim 1, 2, or 3 wherein the measuring step includes determining the degree of cell adhesion to ligands expressed on other cells.

12. The method of claim 1, 2, or 3 wherein the measuring step includes biochemical assays of activity of tyrosine kinase.

13. The method of claim 2, wherein the gene expressing said *volado* protein is from a vertebrate or an invertebrate.

14. The method of claim 3, wherein the gene expressing said integrin protein is from a vertebrate or an invertebrate.

5. A method of detecting a test compound for cognitive enhancer activity, comprising the steps of:

feeding Drosophila flies the test compound; and

testing the treated Drosophila flies for effects on their olfactory learning and memory.

16. The method of claim 15, wherein the Drosophila flies are wild type flies or *volado* mutants.

17. The method of claim 13, wherein the gene is from Drosophila.

18. The method of claim 13 or 14, wherein the gene is from a mammal.

19. The method of claim 16, wherein said *Volado* mutant is selected from the group consisting of $vol^1$ and $vol^2$.

20. An Antibody against a *Volado* protein.

21. An Antibody against an integrin protein.

22. A method of screening for mutant Drosophila flies involved in cognitive processes comprising the steps of:

making a plurality of Drosophila fly line, each line containing a transposable element linked to a reporter gene;

crossing each such line with Drosophila flies containing specific genetic make-up, wherein during each such cross the transposable element is capable of moving the reporter gene to a new site in the genome; and

screening the progeny of such cross for alteration in reporter gene activity in the mushroom bodies.

23. The protein sequence identified in SEQ ID NO. 1.

24. The protein sequence of claim 23 in which the first 63 amino acids are replaced with the 63 amino acids identified in SEQ ID NO. 2.

25. The method of claim 2, wherein the protein sequence is SEQ ID NO.1 or SEQ ID NO. 1 wherein the first 63 amino acids are replaced by SEQ ID NO. 2.

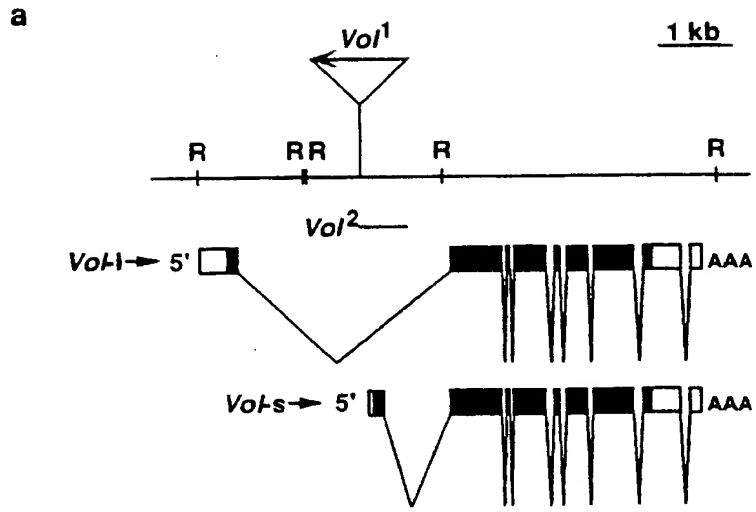


FIG. 1A

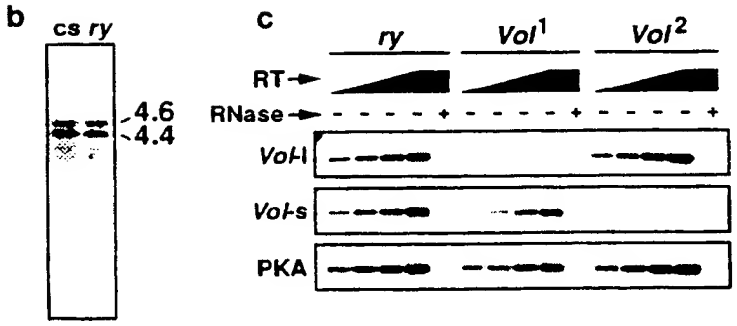


FIG. 1B


FIG. 2A


FIG. 2B


FIG. 2C

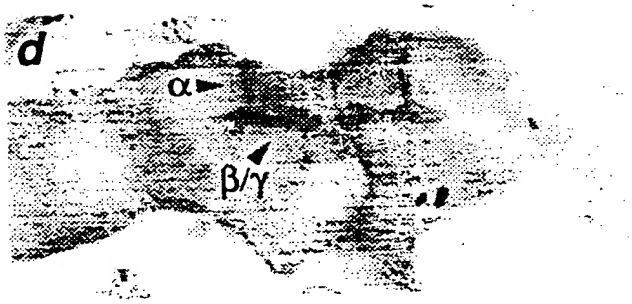


FIG. 2D

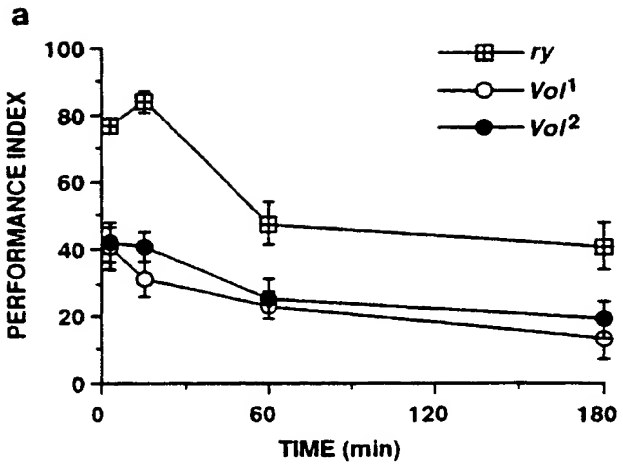


FIG. 3A

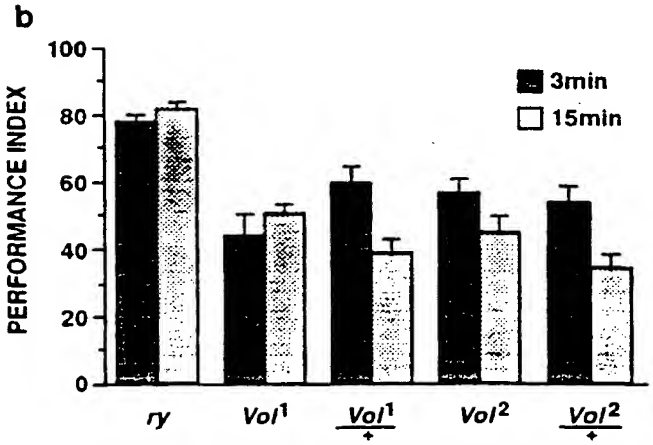


FIG. 3B

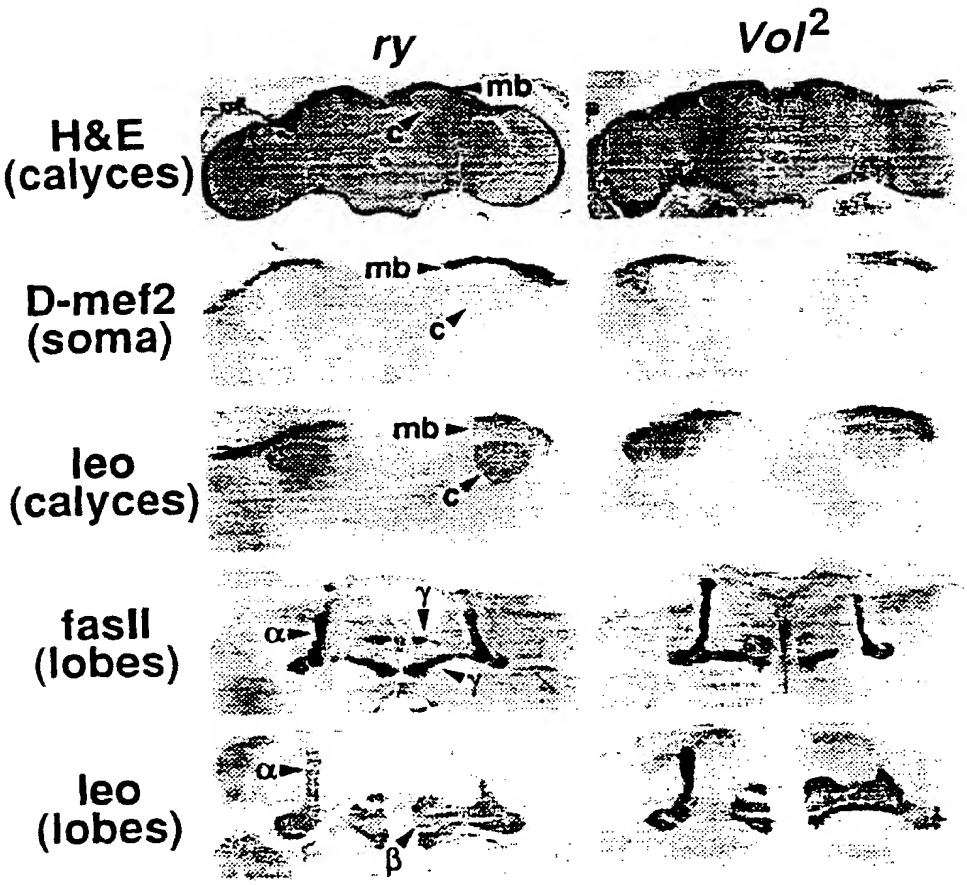


FIG. 4

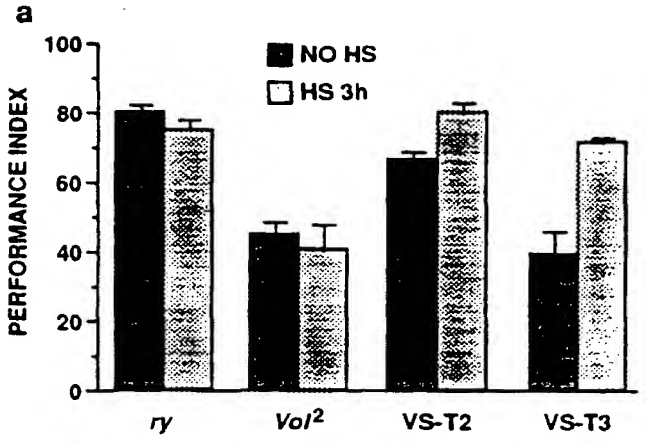


FIG. 5A

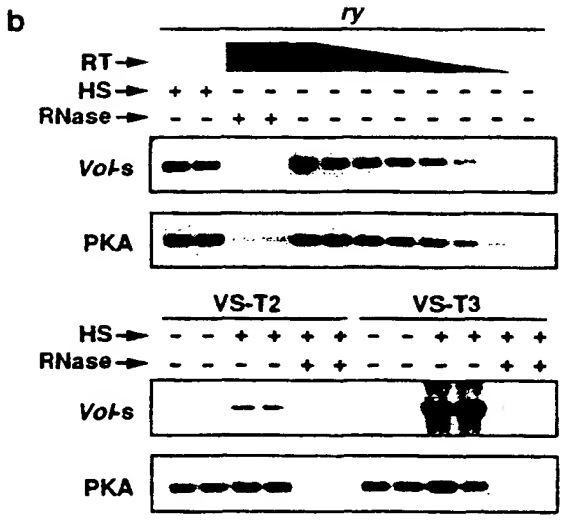


FIG. 5B

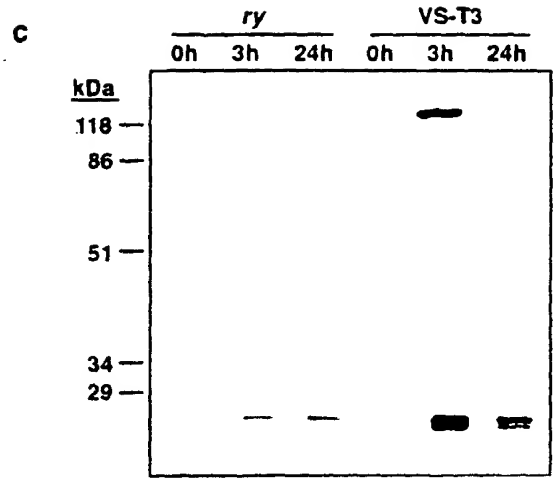


FIG. 5C

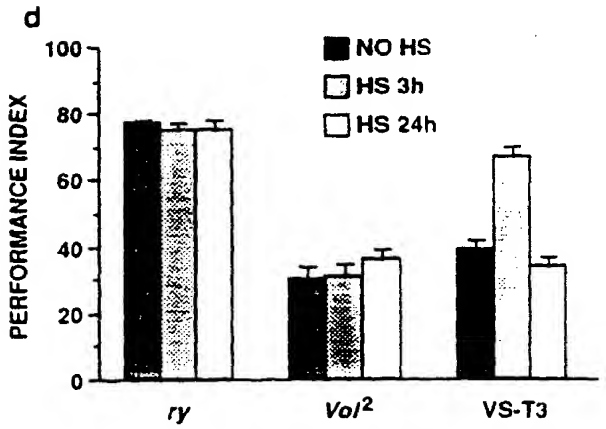


FIG. 5D

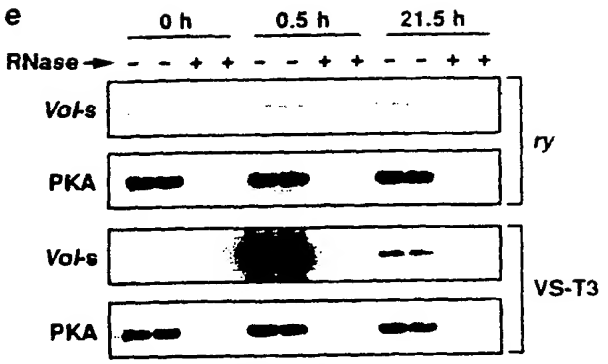


FIG. 5E

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/01592

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C07K 14/00, 16/00; C12N 15/00; C12Q 1/00; G01N 33/53
US CL :435/4, 7.1; 530/350, 387.1; 800/3, 13

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 7.1; 530/350, 387.1; 800/3, 13

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | SKOULAKIS et al. Olfactory learning deficits in mutants for leonardo, a Drosophila gene encoding a 14-3-3 protein. Neuron. November 1996, Vol. 17, pages 931-944, entire document. | 1-25 |
| Y | BEHAN et al. Displacement of corticotropin releasing factor from its binding protein as a possible treatment for Alzheimer's disease. Nature. 16 November 1995, Vol. 378, pages 284-287, entire document. | 1-25 |
| Y | ZHU et al. Volado: A gene encoding a novel α-subunit of integrin which influences learning. Abstracts of Papers Presented at the Meeting on Neurobiology of Drosophila. 1995, page 9. | 1-25 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
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| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 12 APRIL 1999 | 13 MAY 1999 |

| Name and mailing address of the ISA/US | Authorized officer |
|---|---|
| Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | ANNE-MARIE BAKER, PH.D. |
| Facsimile No. (703) 305-3230 | Telephone No. (703) 306-0196 |

JOYCE BRIDGERS
PARALEGAL SPECIALIST
CHEMICAL MATRIX

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/01592

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | HEINRICHS et al. Enhancement of performance in multiple learning tasks by corticotropin-releasing factor-binding protein ligand inhibitors. Peptides. 1997, Vol. 18, No. 5, pages 711-716. | 1-25 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/01592

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS
Dialog (file: medicine)
search terms: volado, drosophila, learning, memory, cognit?, integrin, spodoptera

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/00, 9/02, 15/62
A01K 67/027, C12N 1/19, 9/10
C12N 15/12, 15/53

A1

(11) International Publication Number: WO 92/14817

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(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : WOLF, Charles, Roland [GB/GB]; ICRF Laboratory of Molecular Pharmacology and DrugMetabolism, Department of Biochemistry, Hugh Robson Building, University of Edinburgh, George Square, Edinburgh EH8 9XD (GB). JOWETT, Trevor [GB/GB]; University of Newcastle, Department of Biochemistry & Genetics, Old Medical School, Catherine Cookson Building, Newcastle-on-Tyne NE2 4HH (GB). BEGGS, Jean, Duthie [GB/GB]; University of Edinburgh, Department of Molecular Biology, King's Building, Mayfield Road, Edinburgh EH9 3JU (GB).

(74) Agent: BASSETT, Richard, S.; Eric Potter & Clarkson, St. Mary's Court, St. Mary's Gate, Nottingham NG1 1LE (GB).

(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
*With international search report.*

(54) Title: IN VIVO ASSAY SYSTEMS FOR METABOLIC ROUTES

(57) Abstract

A cellular organism useful in an assay for determining the metabolism of a compound, the organism comprising in the genome of its cell or at least one of its cells a coding sequence for expressing a polypeptide having the function of a naturally-occurring protein which is involved in the alteration of the metabolism, mutagenicity or toxicity of a compound under the regulatory control of a suitable promoter, the combination of the coding sequence and the promoter not normally being found in the said cell of the said organism. Preferably, the protein is a P450 cytochrome-dependent enzyme. The organism may be yeast (in which case a mammalian NADPH:cytochrome P450 reductase or a hybrid yeast/mammalian P450 reductase can usefully be encoded as well), a rodent (in which case expression in the skin using a keratin promoter is preferred, optionally with co-expression of a glutathione S-transferase) or a *Drosophila* fly.

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# IN VIVO ASSAY SYSTEMS FOR METABOLIC ROUTES

The present invention relates to assay systems for determining the metabolic fate of a compound in mammals, particularly in humans. It also relates to transgenic organisms *per se*, useful in these and other contexts.

The manner in which exposure to chemicals in the environment influences the incidence of human disease is a central issue at the present time. The rate of metabolism and disposition of such compounds is of central importance in determining our response to such compounds. These reactions are catalyzed by a broad range of proteins generally termed drug metabolizing enzymes. Individuality in the expression of these proteins may be a critical determinant in susceptibility to diseases such as cancer. On this premise it is of obvious importance to clearly identify the metabolic routes of chemical toxin and carcinogen disposition. A limitation of almost all studies to date, on this theme, has been the lack of *in vivo* models to evaluate the role of specific enzymes in toxicological response.

The cytochrome P450-dependent monooxygenases play a critical role in the metabolism and disposition of foreign chemicals. These enzymes oxidise potentially toxic lipophilic compounds to products which are subsequently conjugated and excreted as water soluble metabolites. Ironically, the enzymes can convert non-toxic compounds to highly reactive chemical intermediates such as epoxides which react with DNA, RNA or protein producing cytotoxic, genotoxic and carcinogenic effects (Wolf, 1986). In view of its critical function in detoxification the cytochrome P450 monooxygenase system has diverged into several multigene families encoding proteins with distinct but overlapping substrate specificities. Because the response of an individual to a particular drug, toxin or carcinogen

could be the product of several different enzyme activities it is extremely difficult to assess the relative contribution of each enzyme in determining the overall effect. The problem is further compounded by there being polymorphisms in the genes involved which, in turn, alter the susceptibilities of individuals to particular chemicals. Variation between individuals also depends upon the previous history of exposure to chemicals or drugs because a number of the genes are inducible by a variety of different chemical agents (Nebert and Gonzalez, 1987; Adesnik and Atchison, 1986). In order to unravel this bewildering complexity of factors a versatile *in vivo* model is required.

The Somatic Mutation and Recombination Test (SMART) (Graf *et al*, 1984; Szabad *et al*, 1983; Szabad, 1986; Frölich and Würgler, 1989) involves exposure of *Drosophila* larvae, heterozygous for different recessive cuticle markers, to a potential mutagen. Clones of marked cells in the imaginal discs are induced by a number of genotoxic effects: somatic mutation, chromosomal rearrangements or nondisjunction. These events are subsequently manifest as mosaics in the differentiated structures of the adult. This is a single generation test which involves exposing larvae to the potential mutagen.

Cytochrome P450-dependent monooxygenases (P450s) are a supergene family of enzymes that catalyse the oxidation of lipophilic chemicals through the insertion of one atom of molecular oxygen into the substrate. They are involved in the metabolism of a vast range of xenobiotic compounds, and in particular with the clearance of almost all pharmaceutical drugs. The P450 system is polymorphic in man, and genetic differences in the P450-mediated metabolism of a wide variety of drugs have been clearly demonstrated. The best example of this is the debrisoquine/sparteine polymorphism. Up to 10% of the

Caucasian population exhibit the poor metaboliser (PM) phenotype. This is characterised by a significantly reduced ability to metabolise the prototype drug debrisoquine to 4-hydroxydebrisoquine, the metabolism being 10-200 times less than in extensive metabolisers (EMs). The PM phenotype is inherited as an autosomal recessive trait, and up to 54% of people are carriers of a mutant allele(s). The PM phenotype leads to impaired clearance of over twenty other commonly prescribed drugs including (in the cardiovascular area) metoprolol, timolol, propranolol, perhexilene, N-propylamaline, propafenone, encainide, flecainide and mexiletine, (in the psychiatric area) amitriptyline, imipramine, desipramine, nortriptyline, clomipramine, thioridazine, perphenazine, amiflamine and tomoxitene and (in other areas) codeine, methoxyphenamine, cyclophosphamide and phenformin and possibly also chlorpropamine, melatonin and MPTP and may result in serious adverse side effects upon their administration. It is thought that almost all carcinogenic agents are converted by P450 enzymes into their ultimate carcinogenic form.

One aspect of the present invention provides a cellular organism useful in an assay for determining the metabolism of a compound, the assay comprising a transgenic cellular organism comprising in the genome of its cell or at least one of its cells a coding sequence for expressing a polypeptide having the function of a naturally-occurring protein which is involved in the alteration of the mutagenicity or toxicity of a compound under the regulatory control of a suitable promoter, the combination of the coding sequence and the promoter not normally being found in the said cell of the said organism.

Preferably, the said naturally-occurring protein is one involved in determining the susceptibility of a cell to a toxic or mutagenic chemical for example mdr (multiple drug

resistance) transport protein, a glutathione-S-transferase, a UDP-glucuronosyl transferase, epoxide hydrolase or an enzyme from the superfamily of P450 cytochrome dependent enzymes. In this specification, for convenience, the terms "P450", "P450 enzyme" and "P450 activity" are used to refer to enzymes of the said superfamily and their activity.

There is one microsomal/mitomondrial form and several cytosolic forms of GST (EC 2.5.1.18). The GST isozymes exhibit overlapping substrate specificities, including alkyl and aryl halides, aromatic amines, lipid hydroperoxides, $\alpha,\beta$-unsaturated ketones, quinones and epoxides.

The P450 gene may be any of those identified in Nebert *et al* (1987) *DNA* **6**, 1-11, Nebert *et al* (1989) *DNA* **8**, 1-13 or any subsequent update.

Present knowledge indicates that each P450 gene usually produces a single protein (the enzyme). To date, there appear to be several exceptions to this rule where "functional" alternative splicing might occur, ie differential processing of the P450 transcript such that entire exons or portions of exons are exchanged in order to produce an enzyme with a new catalytic activity.

There are presently 141 P450 genes and six pseudogenes that have been described in 24 eukaryotes (including ten mammalian and two plant species) and in four prokaryotes (Table B). Of the 26 gene families so far described, ten exist in all mammals. These ten families comprise 19 subfamilies, or clusters of genes, of which 15 and 11 have been mapped in the human and murine genome, respectively (Table A).

**TABLE A: CHROMOSOMAL AND SUBCHROMOSOMAL LOCALISATION OF CYP GENES**

| P450 cluster or gene | Chromosomal location | References |
|---|---|---|
| | | HUMAN |
| **CYP1** | 15q22-qter (near **MPI**) | |
| **CYP2A** | 19q13.1-13.2 | Miles *et al* (1990a) |
| **CYP2B** | 19q12-q13.2 | Miles *et al* (1988) Yamano *et al* (1989) |
| **CYP2C** | 10q24.1-24.3 | Shephard *et al* (1989) |
| **CYP2D** | 22q11.2-qter | |
| **CYP2E** | 10 | |
| **CYP2F** | 19 | Nhamburo *et al* (1990) |
| **CYP2G** | | |
| **CYP3A** | 7q21.3-q22 | |
| **CYP3B** | | |
| **CYP4A** | 1 | O.W. McBride and J.P. Hardwick in preparation |
| **CYP4B** | 1p12-p34 | Nhamburo *et al* (1989) |
| **CYP7** | | |
| **CYP11A** | 15 | |
| **CYP11B** | 8q21 | |
| **CYP17** | 10 | |
| **CYP19** | 15 | |

| | | |
|---|---|---|
| **CYP21** | 6p (within HLA) | |
| **CYP26** | 2q33-qter | J.J. Cali, D.W. Russell, U. Francke *et al* in preparation |

MOUSE

| | | |
|---|---|---|
| **Cyp1** | Mid-9 (near Mpi-1) | |
| **Cyp2a** | 7 (near Gpi-1) | Matsunage, T. *et al* (1990)<br>Miles *et al* (1990b)<br>Burkhart *et al* (1990) |
| **Cyp2b** | Proximal 7 (Coh) | Miles *et al* (1990b) |
| **Cyp2c** | 19 | |
| **Cyp2d** | 15 | Wong *et al* (1989) |
| **Cyp2** | 7 | |
| **Cyp2f** | 7 | Nhamburo *et al* (1990) |
| **Cyp2g** | | |
| **Cyp3a** | 5 | |
| **Cyp3b** | | |
| **Cyp4a** | 4 | Kimura, S. *et al* (1989b) |
| **Cyp4b** | | |
| **Cyp7** | | |
| **Cyp11a** | 9 | Youngblood *et al* (1989) |

| | | | |
|---|---|---|---|
| **Cyp11b** | | | |
| **Cyp-17** | | | |
| **Cyp-19** | | | |
| **Cyp-21** | 17 (within H-2) | | |
| **Cyp-26** | 1 | J.J. Cali, D.W. Russell, U. Francke *et al*, in preparation | |

References are not included in this Table if the work was cited in the Nebert *et al* (1989) update.

**TABLE B: UPDATE OF ALL CYP GENES AND THEIR PRODUCTS**

| Gene symbol | Trivial name | Species | References |
|---|---|---|---|
| **CYP1A1** | c,βNF-B | rat | |
| | $P_1$ | mouse | |
| | $P_1$,c,form 6 | human | |
| | form 6 | rabbit | |
| | IA1 | trout | |
| | Dah1 | dog | |
| | MKah1 | monkey | |
| **CYP1A2** | P-448,d,HCB | rat | |
| | $P_3$, $P_2$ | mouse | |
| | $P_3$, d, form 4 | human | |

| | | | | |
|---|---|---|---|---|
| | | $LM_4$ | rabbit | |
| | | MC4 | hamster | Lai and Chiang (1990) |
| | | Dah2 | dog | |
| 5 | **CYP2A1** | a1,a,3,UT-F | rat | Matsunaga, T. *et al* (1990) |
| | **CYP2A2** | a2,RLM2 | rat | Matsunaga, T. *et al* (1990) |
| | **CYP2A3** | a3 | rat | Kimura, S. *et al* (1989c) |
| | **Cyp2a-4** | 15αoh-1 | mouse | Lindberg *et al* (1989) |
| | | | | Burkhart *et al* (1990) |
| 10 | **Cyp2a-5** | 15αoh-2 | mouse | Lindberg *et al* (1989) |
| | | | | Burkhart *et al* (1990) |
| | **CYP2A6** | 11A3,P450(1) | human | Yamano *et al* (1989a, 1990) |
| | | | | Miles *et al* (1990a) |
| | **CYP2A7** | AFB | human | Fukuhara *et al* (1989) |
| 15 | **CYP2A8** | MC1 | hamster | Lai and Chiang (1990) |
| | **CYP2B1** | b,PB-4,PB-B | rat | |
| | **CYP2B2** | e,PB-5,PB-D | rat | |
| | **CYP2B3** | IIB3 | rat | |
| 20 | **CYP2B4** | LM2,B0,b15,B1,b46 | rabbit | |
| | **CYP2B4P** | (pseudogene) | rabbit | |
| | **CYP2B5** | b52,B2,HP1 | rabbit | |

| | | | |
|---|---|---|---|
| **CYP2B6** | LM2 | human | |
| **CYP2B7** | IIB1 | human | Yamano *et al* (1989b) |
| **CYP2B8** | IIB2 (pseudogene?) | human | Yamano *et al* (1989b) |
| **Cyp2b-9** | pf26 | mouse | |
| **Cyp2b-10** | pf3/46 | mouse | |
| **CYP2B11** | hIIB | human | Miles *et al* (1988) |
| **CYP2B12** | gene IV | rat | Giachelli *et al* (1989) |
| **CYP2B13** | IIB | dog | Graves *et al* (1990) |
| **CYP2C1** | PBc1 | rabbit | Zhao *et al* (1990) |
| **CYP2C2** | PBc2,K,pHP2 | rabbit | |
| **CYP2C3** | PBc3,3b | rabbit | Chan and Kemper (1990) |
| **CYP2C4** | 1-88,PBc4 | rabbit | Zhao *et al* (1990) |
| **CYP2C5** | form 1 | rabbit | |
| **CYP2C6** | PB1,K,PB-C | rat | Kimura, H. *et al* (1989) |
| **CYP2C6P** | (pseudogene) | rat | |
| **CYP2C7** | f,pTF1 | rat | |
| **CYP2C8** | form 1,IIC2,mp-12, mp-20 | human | |
| **CYP2C9** | IIC1,mp-4 | human | Romkes *et al* (1990) |
| **CYP2C10** | mp,mp-8 | human | |
| **CYP2C11** | h,M-1,16α,2c,UT-A | rat | |

| | | | |
|---|---|---|---|
| **CYP2C12** | i,15β,2d,UT-1 | rat | Zaphiropoulos *et al* (1990b) |
| **CYP2C13** | +g | rat | McClellan-Green *et al* (1989) |
| | | | Zaphiropoulos *et al* (1990a) |
| | -g | rat | Yeowell *et al* (1990) |
| **CYP2C14** | pHP3 | rabbit | |
| **CYP2C15** | b32-3 | rabbit | |
| **CYP2C16** | IIC16 | rabbit | Hassett and Omjecinski (1990) |
| ?**CYP2C17** | pB8 | human | Shephard *et al* (1989) |
| ?**CYP2C18** | 254c | human | Romkes *et al* (1990) |
| ?**CYP2C19** | 29c,6b | human | |
| ?**CYP2C20** | 11a | human | |
| ?**CYP2C21** | MKmp13 | monkey | M. Komori, pers. commun. |
| ?**CYP2C22** | DM 1-1 | dog | M. Komori, pers. commun. |
| **CYP2D1** | db1 | rat | Matsunaga, E. *et al* (1989) |
| **CYP2D2** | db2 | rat | Matsunaga, E. *et al* (1989, 1990) |
| **CYP2D3** | db3 | rat | Matsunaga, E. *et al* (1989, 1990) |
| **CYP2D4** | db4 | rat | Matsunaga, E. *et al* (1989, 1990) |
| **CYP2D5** | db5,CMF1b | rat | Matsunaga, E. *et al* (1989, 1990) |
| | | | Ishida *et al* (1989) |
| **CYP2D6** | db1 | human | |
| **CYP2D7** | IID7 | human | Kimura, S. *et al* (1989d) |

| | | | |
|---|---|---|---|
| **CYP2D8** | IID8 | human | Kimura, S. *et al* (1989d) |
| **Cyp2d-9** | 16α,ca | mouse | Wong *et al* (1989) |
| **Cyp2d-11** | cc | mouse | Wong *et al* (1989) |
| **Cyp2d-12** | cd | mouse | |
| **Cyp2d-13** | ce | mouse | |
| **CYP2E1** | j | human | |
| | j | rat | |
| | 3a | rabbit | |
| | MKj1 | monkey | |
| **CYP2E2** | IIE2 | rabbit | |
| **CYP2F1** | IIF1 | human | Nhamburo *et al* (1990) |
| **CYP2G1** | olf1 | rat | Nef *et al* (1989, 1990) |
| | NMb | rabbit | Ding *et al* (1990) |
| **CYP2H1** | pCHP3 | chicken | Hansen and May (1989) |
| **CYP2H2** | pCHP7 | chicken | Hansen and May (1989) |
| **CYP3A1** | pcn1 | rat | |
| **CYP3A2** | pcn2 | rat | |
| **CYP3A3** | HLp | human | |

| | | | | |
|---|---|---|---|---|
| | **CYP3A4** | nf-25,hPCN1,nf-10 | human | |
| | **CYP3A5** | hPCN3 | human | Aoyama *et al* (1989) |
| | **CYP3A6** | 3c | rabbit | |
| | **CYP3A7** | HLp2 | human | Schuetz *et al* (1989) |
| 5 | **CYP3A8** | HFL33 | human | Komori *et al* (1989a, 1989b) |
| | **CYP3A9** | MKnf2 | monkey | |
| | **CYP3B1** | olf2 | rat | |
| 10 | **CYP4A1** | LA 1 | rat | Kimura, S. *et al* (1989a) |
| | **CYP4A2** | IVA2 | rat | Kimura, S. *et al* (1989a) |
| | **CYP4A3** | IVA3 | rat | Kimura, S. *et al* (1989b) |
| | **CYP4A4** | p-2 | rabbit | |
| | **CYP4A5** | KDB3 | rabbit | Johnson *et al* (1990) |
| 15 | **CYP4A6** | R9, KDA6,ka-1 | rabbit | Yokotani *et al* (1989) |
| | | | | Johnson *et al* (1990) |
| | **CYP4A7** | R4, KDB18,ka-2 | rabbit | Yokotani *et al* (1989) |
| | | | | Johnson *et al* (1990) |
| | **CYP4A8** | p-2-like | human | Yokotani *et al* (1990) |
| 20 | **CYP4A9** | PP1 | rat | Stromstedt *et al* (1990) |

| | | | |
|---|---|---|---|
| **CYP4B1** | lung P450 | human | Nhamburo *et al* (1989) |
| | form 5 | rabbit | Gasser and Philpot (1989) |
| | form 5 | rat | Gasser and Philpot (1989) |
| **CYP4C1** | P-450 | cockroach | Y.H. Lee, L.L. Keeley, J.Y. Bradfield, in preparation |
| **CYP6** | VIA1 | house fly | Feyereisen, R. *et al* (1989) |
| **CYP7** | 7α | rat | Noshiro *et al* (1989) |
| | | | Jelinek *et al* (1990) |
| | | | Li *et al* (1990) |
| | | human | Noshiro and Okuda (1990) |
| | | rabbit | |
| | | cow | |
| | | baboon | |
| **CYP10** | P-450 | pond snail | |
| **CYP11A1** | scc | human | |
| | | cow | Ahlgren *et al* (1990) |
| | | pig | Mulheron *et al* (1989) |

| | | | |
|---|---|---|---|
| | rat | Oonk *et al* (1989) |
| **CYP11B1** | 11β | human | Mornet *et al* (1989) |
| | | cow | Kirita *et al* (1988) |
| | | | Hashimoto *et al* (1989) |
| | | rat | Nonaka *et al* (1989) |
| | | | Naomichi *et al* (1990) |
| | | | Imai *et al* (1990) |
| | | | Matsukawa *et al* (1990) |
| **CYP11B2** | (pseudogene?) | human | Mornet *et al* (1989) |
| **CYP17** | 17α | human | |
| | | cow | Bhasker *et al* (1989) |
| | | pig | |
| | | chicken | |
| | | rat | Namiki *et al* (1988) |
| | | | Fevold *et al* (1989) |
| | | | Mellon and Vaisse (1989) |
| **CYP19** | arom | human | Means *et al* (1989) |
| | | | Harada *et al* (1990) |

| | | | |
|---|---|---|---|
| | | rat | Lephart *et al* (1990) |
| | | | Hickey *et al* (1990) |
| **CYP21A1** | c21 | cow | |
| | c21A | mouse | |
| | c21 | pig | |
| **CYP21A1P** | (pseudogene c21A) | human | |
| **CYP21A2** | c21B | human | |
| **Cyp21a-2p** | (pseudogene c21B) | mouse | |
| **CYP26** | 26-ohp | rabbit | Andersson *et al* (1989) |
| | | rat | Usui *et al* (1990) |
| | | | Su *et al* (1990) |
| | | human | |
| **CYP51** | 14DM | *S. cerevisiae* | |
| | | *C. tropicalis* | |
| | | *C. albicans* | Lai and Kirsch (1989) |
| **CYP52A1** | alk1 | *C. tropicalis* | Sanglard and Loper (1989) |
| | | | Sanglard and Fiechter (1989) |
| **CYP52A2** | alk2 | *C tropicalis* | |

| | | | |
|---|---|---|---|
| **CYP52A3** | Cm-1 | *C. maltosa* | Schunck *et al* (1989) |
| **CYP52A4** | Cm-2 | *C. maltosa* | |
| **CYP53** | bphA | *Asp. niger* | R. van Gorcom, pers. commun. |
| **CYP54** | C1-1 | *N. crassa* | Attar *et al* (1989) |
| **CYP55** | dNIR | *E. oxysporum* | Kizawa *et al* (1990) |
| **CYP56** | DIT2 | *S. cerevisiae* | Briza *et al* (1990) |
| **CYP71** | | Avocado | Bozak *et al* (1990) |
| **CYP101** | cam | *Ps. putida* | |
| **CYP102** | BM-3 | *B. megaterium* | Ruettinger *et al* (1989) |
| **CYP103** | pinF1 | *Agr. t'faciens* | Kanemoto *et al* (1989) |
| **CYP104** | pinF2 | *Agr. t'faciens* | Kanemoto *et al* (1989) |
| **CYP105A1** | SU1 | *Str. griseolus* | Omer *et al* (1990) |

| | | | |
|---|---|---|---|
| **CYP105B1** | SU2 | *Str. griseolus* | Omer *et al* (1990) |
| **CYP105C1** | choP | *Str. sp.* | Horii *et al* (1990) |
| **CYP106** | BM-1 | *B. megaterium* | He *et al* (1989) |

The References are listed in Nebert *et al* (1991) *DNA & Cell Biol.* **10**(1), 1-14. References are not included in this Table if the work was cited in the Nebert *et al* (1989) update. Okada *et al* (1989) reported a plant oxidase cDNA sequence exhibiting similarities to the $NH_2$ terminus of the mouse Cyp1a-1 protein; since this gene does not have the cysteinyl-containing heme-binding site in the COOH terminus, it does not qualify as a member of the P450 gene superfamily.

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In one embodiment, the organism is a eukaryote, such as a *Drosophila* (eg *D. melanogaster*), a rodent (eg a rat or a mouse) or a yeast (eg *Saccharomyces cerevisiae*). In a multi-cellular organism, the said promoter preferably provides for expression in only some cell types of the organism, for example the fat body (the insect equivalent of the liver) of the third instar *Drosophila* larva (eg using the *Drosophila* LSP1α gene promoter) or the skin of a rodent (eg using the bovine keratin VI promoter). However, in the case of short-lived putative carcinogens, it may be appropriate to provide for expression in most or all tissues of the host. In *Drosophila*, for example, this can be achieved with the Hsp70 promoter. When the promoter is such as to cause expression of the P450 enzyme in only some cells of the organism or in only some developmental stages of the organism, then clearly the organism is used in the assay methods of the invention such that the test compound is applied directly or indirectly to the cells or at the relevant developmental stage.

At least in lower eukaryotes such as yeast, and also in prokaryotes such as *Salmonella* spp., it may be found that the natural mammalian P450 enzyme does not function adequately. Hybrid enzymes can be constructed to provide a region having mammalian (eg human) P450-type enzymatic specificity and a region having the requisite lower eukaryote or prokaryote function. For example, the polypeptide can be so encoded as to have a human P450 enzyme region and a region from the yeast P450 enzyme which will bind yeast membranes and thereby increase the enzymatic activity of the polypeptide in yeast. Alternatively, the portion of the mammalian P450 enzyme which does not allow adequate expression in the lower eukaryote or prokaryote (for example the hydrophobic amino terminal amino acids) may simply be deleted.

It is necessary for the mammalian P450 enzyme to be expressed with the co-enzym NADPH:cytochrome P450 reductase. In some hosts, for example lower eukaryotes and prokaryotes, the endogenous reductase enzyme may not be present or may not form a functional relationship with the mammalian P450 monooxygenase. One solution is to co-transform the organism with a gene for a suitable (eg mammalian) co-enzyme, another is to express a hybrid, fusion protein comprising the enzymatically active portions of the P450 enzyme and mammalian reductase and a third is to express a similar fusion protein but using the host's reductase, for example bacterial or yeast reductase.

Thus, the fusion protein of the invention may comprise a first region providing the activity of a naturally-occurring protein from a first organism which protein is involved in the alteration of the mutagenicity or toxicity of a compound and a second region adapted to bind to cell membranes, the second region being homologous to a cell-binding region of a protein in an organism other than the first organism.

Preferably, the first organism is a mammal and the said naturally-occurring protein is the mdr transport protein, a glutathione S-transferase, P450 reductase or an enzyme of the superfamily of P450 cytochrome dependent enzymes.

Suitably, the second region is adapted to bind to yeast cell membranes.

Advantageously, the fusion protein comprises the sequence $H_2N-R^1-R^2-R^3-COOH$ wherein $R^1$ is homologous to the n N-terminal amino acids of *Saccharomyces cerevisiae* P450 reductase where n is 10-30, $R^2$ is homologous to the n' N-terminal amino acids of human P450 reductase where n' is 10-56, and $R^3$ provides the enzymatic function of human P450 reductase.

By "homologous" we mean that the two sequences being compared have at least 30% identity of amino acids, preferably at least 50%, 75%, 90%, 95% or 99% identity. Preferably, $R^3$ comprises the soluble portion of mammalian P450 reductase or a homologous amino acid sequence, for example the 56-678 region.

The main purpose of attaching the transgenic product to the host's cell membranes is to bring the P450 enzyme and the P450 reductase into mutual proximity. The same end effect may be achieved without attachment to membranes if the two enzymes are expressed as a fusion protein. Thus, a further molecule of the invention comprises a first region having the enzymatic function of a mammalian P450 enzyme and a second region having the enzymatic function of a P450 reductase, preferably a mammalian P450 reductase.

The said enzymatic function of the P450 enzyme is to oxidase substrates as described above and it will normally bind haem (present in yeast) to do this. The enzymatic function of the reductase is to channel electrons to the P450 enzyme and it will normally bind one molecule each of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to do this. Thus, the said first region will normally (a) bind haem, and, when haem is bound, (b) bind a P450 enzyme substrate such as one or more of those listed above, (c) react with carbon monoxide to give the characteristic 450 nm optical band and (d) oxidise the said substrate. It is typically 50-60 kDa in molecular weight. The second region will normally have NADPH-dependent cytochrome c reductase activity and is typically 60-70 kDa in molecular weight. The sequence of each region may be derived from the corresponding sequences of P450 and reductase, omitting any hydrophobic terminal region which, in the native molecule, serves to bind membranes.

Non-enzymatically active linker and terminal extension

regions may be included if desired. The said first region may be N-terminal or C-terminal to the said second region.

Particular fusion proteins include the following:

| | First Region | Second Region |
|---|---|---|
| (i) | full length mammalian (preferably human) P450 enzyme | full length mammalian (preferably human) reductase |
| (ii) | full length mammalian (preferably human) P450 enzyme | full length *Bacillus megaterium* reductase |
| (iii) | full length mammalian (preferably human) P450 enzyme | mammalian (preferably human) reductase, less hydrophobic domain |
| (iv) | mammalian (preferably human) P450 enzyme, less hydrophobic domain | full length mammalian (preferably human) reductase |
| (v) | mammalian (preferably human) P450 enzyme, less hydrophobic domain | full length *Bacillus megaterium* reductase |
| (vi) | mammalian (preferably human) P450 enzyme, less hydrophobic domain | full length *Bacillus megaterium* reductase |
| (vii) | *Bacillus megaterium* P450 domain | full length mammalian (preferably human) reductase |
| (viii) | *Bacillus megaterium* P450 domain | full length *Bacillus megaterium* reductase |
| (ix) | *Bacillus megaterium* P450 domain | full length *Bacillus megaterium* reductase |

In each case, the first region is preferably N-terminal to the second region.

In the truncated mammalian reductase (rat or human), regions of the hydrophobic membrane anchor will be removed ie amino acids 1-56. For the truncated P450s, the

hydrophobic tail will be removed between amino acids 1-50.

A linker may be included between the first and second regions. The size of the linker region may be in the order of 6-20 amino acids. The *B. megaterium* linker sequence(s) or a similar hydrophilic linker may be used. (The linker region of the *B. megaterium* fusion contains approximately 20 amino acids of which 8 of these are charged and 7 are hydrophilic). The linker preferably incorporates a multiple cloning site to facilitate a variety of different P450 cDNA sequences. The *Bacillus megaterium* P450/reductase molecule is described in Li *et al* (1991), Narhi *et al* (1987) and Ruettinger *et al* (1989). These fusion compounds may be expressed in any suitable host (eg *Salmonella*) if they are soluble.

It may be advantageous for the cells in which the P450 activity is expressed to have the glutathione S-transferase (GST) activity deleted. GST is a general detoxifying enzyme and it may be informative to study the effect of the P450 activity on the compound without GST interfering with the compound's P450 metabolism. Alternatively, especially in non-mammalian hosts expressing mammalian P450 activity, it may be advantageous to co-express mammalian GST precisely to mimic the normal human situation. When doing this, if the host tissue has a significant level of endogenous GST, it may be advantageous to delete the host's GST gene(s) so that only the transgenic GST is expressed. The *Drosophila GST* genes have been described in Toung *et al* (1990) and it is possible to select for P element insertions which prevent GST gene expression using known methods, for example those of Ballinger & Benzer (1989) or Kaiser & Goodwin (1990). Alternatively, or as well, the level of transgenic protein (for example GST) may be increased (in absolute terms or in relation to endogenous protein, if present) by inserting more than one copy of the transgene.

Transgenic organisms expressing useful levels of mammalian P450 enzyme activity as indicated above are novel and form a further aspect of the invention, whether or not they are used in the assay systems of the first aspect of the invention. Preferably, the organism is a higher eukaryote or a prokaryote. Although attempts have previously been made to express mammalian P450 enzymes in yeast, these have only produced low levels of P450 activity. By deleting the hydrophobic tail of the enzyme and optionally replacing it with a hydrophobic tail from a host (yeast) enzyme, useful levels of mammalian P450 activity can be obtained. "Higher eukaryotes" in this specification means multicellular eukaryotes, such as insects, fish, birds and mammals.

The invention also encompasses methods of making the transgenic organisms by transfection or transformation with the polypeptide-encoding sequence and an appropriate promoter; isolated constructs comprising the polypeptide-encoding sequence and a promoter which is (i) heterologous to the coding sequences (ie not naturally found in regulatory association therewith) and (ii) effective in a higher eukaryote; coding sequences for the said hybrid enzymes; and the hybrid enzymes themselves.

A further aspect of the invention provides a method of assessing the toxicity of a compound, the method comprising exposing an organism as described above to the compound and determining either the metabolism of the compound or the effect of the compound on the organism, its development or its progeny.

The organisms of the invention may be used in conjunction with the toxicity screening methods of, for example, EP 289 121, US 4 753 874, WO 89/05864, WO 89/09272, EP 370 813, Oda *et al* (1985 *Mutat. Res.* **147**, 219-229) or Hall *et al* (1983 *J. Mol. App. Genet.* **2**, 101-109). Thus, the host organism of those various systems, particularly where it is prokaryotic or

lower eukaryotic, can be improved by engineering it to express a human P450 enzyme or other protein in accordance with the invention, so that the assay gives a better indication of what really happens in a human exposed to the compound being screened.

The transgenic organisms, particularly prokaryotes such as *E. coli* and *Salmonella spp.*, may also be used to degrade environmentally harmful compounds to less harmful compounds and may therefore be used to detoxify industrial waste and spillages of, for example, oil. Such organisms may contain transgenes with other compound-degrading capabilities or may be used in conjunction with other organisms to provide a broad spectrum of degradative capabilities.

Aspects of the invention will now be illustrated by way of example (all references being incorporated herein by reference) and with reference to the accompanying drawings in which the figure legends are as follows:

**Figure 1.** Structure of the transgene constructs introduced into the *Drosophila* germline.

**Figure 2.** Breeding scheme for selection of transformants. SM1, Cy: second chromosome balancer carrying dominant Curly wings marker. TM3, Sb $ry^{RK}$: third chromosome balancer with dominant Stubble bristle marker has a mutant rosy gene. $\alpha^{F}$: LSP1alpha fast electrophoretic variant. $\beta^{o}$: LSP1beta null allele. $\Gamma^{o}$ 2' ry: LSP1gamma deficiency, LSP2 slow electrophoretic variant, rosy.

**Figure 3.** Genomic Southern of DNA from each of the two *alacZ* transformed lines and the two *αCYP2B1* lines. The DNA was digested with *Eco*RI and probed with an *Hind*III fragment from the 5' end of the $rosy^{+}$ gene in Carnegie 30. This fragment identifies genomic fragments of different sizes according

to the position of the nearest *Eco*RI site at the point of integration of the construct. The common band is from the $ry^{506}$ gene originally present in the host strain.

**Figure 4.** Northern analysis of total RNA from fat bodies (F) and remaining carcass (C) of third instar larvae of each of the four transformed lines (B3 and K2 carry *alacZ*, C1 and D1 carry an *αCYP2B1* insertion). Each track contains the RNA from half a larval equivalent. Three identical filters were produced and each probed separately with the *CYP2B1* *Eco*RI fragment (left), the *lacZ* *Eco*RI fragment containing *Adh*, *lacZ* and SV40 sequence (middle), and a 200bp *Bam*HI fragment from the start of the *LSP1α* gene. Z, P, A and α denote the *αlacZ*, cytochrome P450, alcohol dehydrogenase and *LSP1α* transcripts respectively.

**Figure 5.** Third instar fat bodies stained for β-galactosidase activity or for CYP2B1 protein. (A) *αlacZB3* fat body stained blue with X-gal. (B) *αCYP2B1* fat body stained brown showing localisation of the cytochrome P450 to fat body cells. The salivary glands and testes remained colourless.

**Figure 6.** Identification of the expressed CYP2B1 protein by Western blotting. (a) Extracts from whole *Drosophila*. C, purified CYP2B1 from rat liver microsomes (control); E3, early third instar larva; M3, mid third instar larva; L3, late third instar larva; P, pupa; A1, 4 h old adult; A2, 24h old adult; H, mid third instar larva of untransformed host strain. (b) Localisation of the CYP2B1 protein to the microsomal fraction of transformed third instar larvae. M, microsomes; C, cytosol; P, 0.5pmol of purified CYP2B1 from rat liver.

**Figur 7.** Breeding schemes to introduce the transgenic

constructs inserted on chromosome II into the genetic backgrounds for performing the SMART assay. B$^s$Y: derivative of Y chromosome with dominant B$^s$ mutation that causes extreme Bar eyes. C1B: first chromosome balancer with dominant Bar eye marker, recessive lethal therefore males die. e: ebony body. flr$^3$: abnormal trichomes and bristles, recessive zygotic lethal but viable as small clones of homozygous cells. fs(1)K10: abnormal egg shape when homozygous in germ line cells. mwh: multiple wing hair, 2-7 processes instead of 1 per wing blade cell. se: dark brown eyes to facilitate recognition of eye mosaic spots. TM2,Ubx se e$^s$: third chromosome balancer with recessive lethal, Ultrabithorax, which causes enlarged halteres. w:white eye. w$^{cc}$:coral red eyes. y: yellow body. y$^+$Y: translocation of y$^+$ allele to the Y chromosome.

**Figure 8.** A 92mer oligo corresponding to the sequence for the 24 n-terminal amino acids of the *S. cerevisiae* P450 reductase (Yabusaki *et al* 1988) was synthesised such that *Hin*dIII and *Sal*I sites were created at the ends. This oligo was made double stranded using a complementary oligo and PCR, digested with *Hin*dIII and *Sal*I and cloned into the polylinker of pTZ18R to create pTZF1.

The variable regions of the hybrid P450 reductase cDNA's were synthesised by PCR using Primer 1 which hybridises at the *Sac*I site within the rat P450 reductase cDNA and either Primer 2, hybridising at the Lys-Arg Trypsin sensitive junction of rat P450 reductase cDNA or Primer 3, hybridising at the n-terminal region of the rat P450 reductase cDNA. PCR was performed using a Thermal Reactor supplied by Hybaid, Teddington, UK according to manufacturer's instructions using *Taq* polymerase supplied by IBI Ltd, Cambridge, UK. The sequences of the fragments synthesised by PCR were confirmed by DNA sequencing. The subsequent products of the PCR were digested with *Sac*I and

*Sal*I and ligated into *Bam*HI/*Sal*I dig sted pTZF1 along with the *Bam*HI/*Sac*I fragm nt of rat P450 r ductase cDNA from pJLF1 (Bligh *et al* 1990) to create pTZF2 and pTZF3. All DNA sequencing and manipulations were by standard techniques (Maniatis *et al* 1982) except where stated. Abbreviations: B = *Bam*HI, H = *Hin*dIII, Sc = *Sac*I, Sl = *Sal*I.

**Figure 9.**

a) pFBY5 and pFBY6 were constructed by ligating the *Hin*dIII P450 reductase encoding fragments from pTZF2 and pTZF3 respectively into the unique *Hin*dIII site of pAAH5 (Ammerer (1983)) such that the orientation was correct for expression from the *S. cerevisiae ADH1* promoter. This plasmid has a *LEU2* selectable marker. pAAH5 was provided by Sandra Jaeger Thompson.

b) pFBY8 was constructed by removing the *Bam*HI fragment containing the hybrid P450 reductase cDNA between the *ADH1* promoter and terminator from pFBY6, and ligating it into the unique *Bam*HI site of pMA56 (Ammerer 1983). This leaves a unique *Eco*RI site available downstream of a second *ADH1* promoter into which a P450 cDNA can be cloned. This plasmid has a *TRP1* selectable marker.

c) pFBY7 and pFBY9 were constructed by ligating a rat P450IIB1 cDNA (Black *et al* 1989) and a human P450 IIA6 cDNA (Miles *et al* 1990) respectively into the *Eco*RI site of pFBY8 such that they were expressed from the *ADH1* promoter.

d) pFBY3 was constructed by ligating a rat P450 reductase cDNA on a *Hin*dIII fragment into the *Hin*dIII site of pAAH5 such that it was expressed from the *ADH1* promoter.

e) pFBY10 was constructed by ligating an *Eco*RI fragment containing the human P450IIA1 cDNA into the *Eco*RI site of pMA56 such that orientation was correct for expression from the *ADH1* promoter.

Abbreviations: B = *Bam*HI, E = *Eco*RI, H = *Hind*III, Sc = *Sac*I, Sl = *Sal*I, *ADH1* = Alcohol Dehydrogenase 1.

Key solid bar = P450 reductase encoding cDNA,
hatched bar = P450 encoding cDNA

**Figure 10.** Hybrid P450 reductase proteins as encoded by constructs illustrated in Figures 8 and 9.

a) Complete rat P450 reductase protein encoded by pFBY3.

b) Hybrid protein encoded on pTZF2 and pFBY5 consisting of the 24 membrane binding amino acids from the endogenous yeast P450 reductase, fused via an Asp residue to the soluble portion of the rat P450 reductase.

c) Hybrid P450 reductase protein encoded on pTZF3 and pFBY6 consisting of the 24 membrane binding amino acids from the endogenous yeast P450 reductase, fused via an Asp residue to the entire rat P450 reductase protein. This hybrid protein was also encoded on pFBY7, pFBY8 and pFBY9 as it was found to be the most stable of the three proteins.

**Figure 11.** Panel A: Western blot of microsomes prepared from S150-2B transformed with 1:pAAH5, 2:pFBY3, 3:pFBY5, 4:pFBY6, 5:pFBY8. Transformations were performed by the Lithium Acetate method (Ito *et al* 1983). Microsomes were prepared by harvesting 1 litre of cells in late log phase at 5000 rpm in a Sorval Superspeed centrifuge, resuspending cells in 50 mM $KPO_4$ buffer (pH7.4) containing 30 mM Dithiothreitol, 1.2M sorbitol and 60 units $ml^{-1}$ lyticase and incubating at 30°C until cells were osmotically fragile. Cells were then washed three times in 1.2M sorbitol and then lysed in 0.1M Tris (pH7.5) containing 50 $\mu g$ $ml^{-1}$ N$\alpha$-P-Tosyl-L-lysine chloromethyl ketone, 100 $\mu g$ $ml^{-1}$ N-Tosyl-L-phenylalanine ketone by brief sonication (1 min X 60W). D bris and mitochondria were removed by centrifugation at 10 g for 10 min and the microsomal fraction isolated by centrifugation of the resultant supernatent at 38K for 1h in a Sorvall OTD55B ultracentrifuge. Pellets were

resuspended in 0.1M $KPO_4$ (pH7.4) buffer containing 20% glycerol 1 mM EDTA and 0.1% glutathione at a protein concentration of 10-20 mg $ml^{-1}$. SDS-PAGE was performed by the method of Laemmli (1970) and Western blotting performed by the method of Towbin *et al* (1979) using goat anti reductase IgG (Wolf *et al* 1979) and a peroxidase-conjugated rabbit anti-goat IgG Vectastain colour detection kit (Vector Laboratories, Bretton, Peterborough, UK). Tracks 7 and 8 contain 360 ng and 720 ng of purified rat P450 reductase as positive control. Arrows indicate proteins of molecular weights a) 80 807, b) 78 225, c) 73 301. Panel B: Northern blot of S150-2B transformed with 1:pAAH5, 2:pFBY3, 3:pFBY5, 4:pFBY6, 5:pFBY8 and probed with 745 nt $^{32}P$ labelled P450 reductase fragment (see Fig 8). RNA was prepared by the method of Schmidt *et al* (1990) and approximately 20 $\mu$g RNA loaded per track. RNA gel electrophoresis was performed as described by Thomas (1983) and blotted onto Hybond N membrane (Amersham Int. plc, Bucks., UK). Prehybridisation, hybridisation and washing were performed as described by Thomas (1980). For standardisation the same amount of total RNA was hybridised to a $^{32}P$ labelled *Eco*RI fragment carrying the *S. cerevisiae* actin gene supplied by Dr D Jamieson. $^{32}P$ labelling was performed as described by Feinberg and Vogelstein (1983).

**Figure 12.** Western blot of microsomal samples prepared from S150-2B transformed with 1:pMA56, 2:p56/3a, 3:pFBY6, 4:pFBY7, 5:pFBY8, 6:pFBY9, 7:pFBY10 (Figure 2). Lane 8 contains 720 ng of purified rat P450 reductase. Samples were prepared as described in Figure 4 and blots were prepared, probed and developed as described in Figure 11. Arrows indicate protein of molecular weights a) 80 807 and b) 78 225. pMA56 was provided by Ben Hall and is described by Ammerer (1983) and p56/3a contains a rat P450IIB1 cDNA and is described by Black *et al* (1989).

**Figur 13.** Panel A: Western blot of microsomal protein (20 μg per track) prepared as described previously (Figure 11) from S150-2B transformed with 2:pMA56, 3:p56/3a, 3:pFBY7, 4:pFBY8 (Figure 2) and probed with rabbit anti rat P450IIB1 antiserum (Black *et al* 1989). Lane 1 contains 50 ng purified rat P450IIB1 protein (Black *et al* 1989). Panel B: Western blot of microsomal protein (40 μg per track) prepared as described previously (Figure 4) from S150-2B transformed with 1:pMA56, 2:pFBY8, 3:pFBY9, 4:pFBY10 (Figure 2) and probed with rabbit anti mouse P450IIA1 antiserum (Miles *et al* 1990). Lane 5 contains 7.5 μg human microsomes as a positive control (Miles *et al* 1990).

Both blots were developed using $^{125}$I labelled protein A as described by Black *et al* (1989).

**Figure 14** is a diagram of vector pMP172 and its construction, in which the multiple cloning site (MCS) includes, in the 5′-3′ direction, *Eco*RV, *Kpn*I, *Sst*I, *Eco*RI and *Bam*HI sites. Repeated flanking rare restriction sites (*Not*I, *Nae*I and *Sfi*I) are shown.

**Figure 15** shows the bovine keratin VI promoter construct used in Example 6. This uses a 4.8 kb fragment of the 5′ end of the gene incorporating the promoter. Because of the rare restriction sites, the entire construct can be removed as one contiguous piece of DNA for generation of the transgenic animals.

**Figure 16.** Effect of skin-specific expression of αGST in transgenic (TG) mice on the level of B(a)P- and DMBA-induced DNA adducts in the skin (A) and lungs (B), following the topical application of 1μmol of each compound.

**Figure 17** shows the functional regions of P450 reductase.

**EXAMPLE 1: TRANSGENIC *DROSOPHILA*: METABOLISM OF P450 SUBSTRATES**

It is the aim of this present study to demonstrate the effectiveness of transgenic *Drosophila* as a powerful approach for studying the metabolic routes of toxins and carcinogens.

The *Drosophila in vivo* system described herein has been found to be one where the genetic background can be controlled, to allow the measurement of a variety of different cytotoxic and genetic endpoints, and to allow assessment of the relative contributions of, and interactions between, different drug metabolizing enzymes. Finally, the transgenic *Drosophila* system has the important advantage that it allows a number of different genetic endpoints to be measured.

In order to determine the effect of a particular enzyme in determining the susceptibility towards the mutagenic properties of a drug, it is necessary to ensure that the enzyme is present at the time the drug is administered. The *Drosophila* promoter chosen was that of the LSP1α gene. This contains all the sequences necessary to confer high levels of expression in the fat body of the third instar larva (Jowett, 1985; Delaney *et al*, 1987). The fat body contains the ovary. The mammalian gene chosen was CYP2B1 (Wolf, 1986; Adesnik and Atchison, 1986; Doehmer *et al*, 1988), a rat gene encoding one of the main phenobarbital-inducible enzymes (P450IIB1 Nebert *et al*, 1987; 1989). As a control, the same promoter fragment was fused to the *E. coli* lacZ gene.

**MATERIALS AND METHODS.**

Gene constructs

The LSP1α promoter was derived from a series of Bal31 exonuclease deletions of a larger genomic fragment (Jowett, 1985). Its proximal end was the *Apy*I site immediately upstream to the first ATG of the protein encoding region. This was converted to an *Eco*RI site by addition of a linker, thereby allowing introduction of cDNA sequences flanked by *Eco*RI sites. The CYP2B1 cDNA was derived from a clone obtained from Dr Milton Adesnik and consisted of 2bp of 5′ untranslated sequence, the entire protein encoding region, 90 bp of 3′ sequence joined to 140 bp of SV40 DNA containing a poly(A) addition signal. This was inserted adjacent to the LSP1α promoter as an *Eco*RI fragment. The lacZ gene was taken from the plasmid pCa⁴hsneoAUGβ-gal provided by Dr Vince Pirrotta and Dr Carl Thummel. The lacZ gene was excised with *Sma*I and *Eco*RI, the *Sma*I site converted to *Eco*RI by addition of a linker and then the result was inserted downstream of the LSP1α promoter. The lacZ *Eco*RI fragment so produced had 120bp of sequence from the start of the Adh gene from *Drosophila* which provided the ATG start of the protein encoding region fused in frame the β-galactosidase encoding region. The 3′ termination and polyadenylation signal was a fragment of SV40 DNA. Both the αCYP2B1 and αlacZ constructs were inserted between the *Not*I and *Sal*I sites of Carnegie 30, in the opposite orientation to the rosy⁺ gene.

P element-mediated transformation.

The host strain for transformation was LSP1α$^{F}$; LSP1β$^{o}$; LSP1Γ$^{o}$LSP2′ry$^{506}$. The transgenes were coinjected with pPi25.7wc as helper as described previously (Jowett, 1985). The selection of transformants and mapping of inserts was performed according to scheme in Figure 2. Southern blot

analysis was performed on single flies once they had been successfully mated, allowing early determination of the number of insertions in each transformed line. Flies carrying single insertions on chromosome II were selected and made homozygous.

Nucleic acid extractions and filter hybridisations

DNA was extracted from several flies or from single flies according to the methods described by Jowett (1986). DNA was digested with restriction enzyme and electrophoresed in 1x TBE buffer (90 mM Tris, 2.5 mM EDTA, 90 mM boric acid). Size markers were end-labelled fragments of a 1 kb ladder (BRL). Gels were soaked in 0.25M HCl for 15 min, 0.5M NaOH, 1.5M NACl for 30 min, 1M ammonium acetate for 30 min and blotted by capillarity in the same buffer for 4h.

RNA was extracted separately from dissected fat bodies and the remaining carcass as described by Jowett (1985). RNA samples were heated to 65°C for 15 min in 20 mM boric acid, 0.8 mM EDTA, 6% formaldehyde, 60% deionised formamide and 0.05 μg/μl ethidium bromide. 1/10 vol 50% glycerol with xylene cyanol and bromophenol blue dyes was added prior to electrophoresis. The size markers were the same end-labelled fragments as for DNA gels but they were first heated to 100°C in 60% formamide prior to mixing with the formaldehye and loading dyes. Electrophoresis was through 1% agarose in 20 mM boric acid, 0.8 mM EDTA overnight at 2V/cm with a magnetic stirrer at the anode. The gel was rinsed 2x 5 min in water and soaked 45 min in 1M ammonium acetate prior to blotting to nitrocellulose in the same buffer.

Nitrocellulose filters were baked at 80°C for 2 h prior to prehybridisation at 65°C in 3x SCP (20x SCP: 2M NaCl, 0.6M $Na_2HPO_4$, 0.02M EDTA) 0.5% sarcosyl, 100μg/ml denatured herring sperm DNA. Heat denatured random-primed $^{32}P$-

labelled probes were added to the filters and prehybridisation solution and left overnight at 65°C. Filters were washed in 2x SCP, 1% SDS at 55°C and then in 3 mM Tris-HCl pH 8.0 at room temperature 2x 30 min. Autoradiography was with Fuji RX film with intensifying screens at -80°C.

*In situ* localisation of the transgenic protein products

β-galactosidase activity was shown by dissecting third instar larvae in phosphate buffered saline (PBS: 130 mM NaCl, 7 mM $Na_2HPO_4$, 30 mM $NaH_2PO_4$), fixing with 4% paraformaldehyde in 1xPBS for 30 min and then incubating in 3 mM potassium ferricyanide, 3mM potassium ferrocyanide, 10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 0.2% X-gal. Fat bodies from the αlacZ transformants developed a deep blue colour in 5-10 min.

The cytochrome CYP2B1 protein was localised using a rabbit antisera raised against the purified protein from rat microsomes. Third instar larvae were dissected in 1x PBS and the tissues fixed with 4% paraformaldehyde in 1xPSB overnight at 4°C. The tissues were soaked briefly 2 min in 100% methanol and then washed in 75% methanol/fix 10 min, 50% methanol/fix 10 min, 25% methanol/fix 10 min, 1xPBS 10 min, and for 10 min in 1x PBT (1xPBS, 0.2% BSA, 0.1% Triton X-100). They were then blocked in PBT+10% goat serum for 60 min. The rabbit antiserum used was preabsorbed with dissected larvae of the αlacZ strain which had been fixed and treated as above. The antiserum was diluted 1 in 200 with PBT+5% goat serum and 25 larvae were incubated overnight at 4°C with gentle agitation in a total volume of 200μl. The preabsorbed antiserum was diluted a further 5x with PBT+5% goat serum and then incubated with the tissues from αCYP2B1 larvae for 2h. The tissues were rinsed twice with PBT, washed 3x 20 min with PBT and incubated 30 min in PBT+5% goat serum. They were then incubated for 60 min

with the second antibody, goat anti-rabbit conjugat d with horseradish peroxidase (Zymed) diluted 1 in 2000 with PBT+5% goat serum. The tissues were again rinsed twice in PBT and then 3x 20 min in PBT before incubating 15 min in PBT containing diaminobenzidine at 0.3mg/ml prior to adding hydrogen peroxide to a final concentration of 0.03%. The brown colour was alloed to develop for 10-15 min in the dark before washing once again in PBT.

Preparation of microsomes, enzyme assays and Western blotting

*Drosophila* microsomes were prepared by homogenising 4g of mid-third instar larvae in 15 ml 1.15% KCl, 10 mM potassium phosphate pH7.4, 0.1% phenylthiourea. The homogenate was centrifuged at 1000g for 5 min and the supernatant respun at 10,000g for 20 min. The remaining supernatant was respun at 100,000g for 1h. The final supernatant was the cytosolic fraction and the pellet, after resuspension in homogenisation buffer without phenylthiourea, was the microsomal fraction.

Fluorometric assays for activities towards the three resorufin homologues were performed as described by Burke & Mayer (1974). SDS polyacrylamide electrophoresis and blotting was performed according to the method of Towbin *et al* (1979).

Construction of the gene fusions

The LSP1α promoter consisted of 1090 bp of the genomic sequence immediately 5′ to the start of the protein encoding region of the LSP1α gene. This was joined to a full length cDNA of the CYP2B1 gene containing 2 bp of untranslated leader sequence, the full protein encoding region and 90 bp of 3′ untranslated region and a 140 bp fragment containing an SV40 polyadenylation signal (Doehmer

*et al*, 1988). A similar construct was made with the same promoter fragment fused to the lacZ gene. This had a 120 bp fragment containing the AUG from the Adh gene of *Drosophila* fused to the lacZ gene with a fragment containing an SV40 polyadenylation signal sequence 3'. Both constructs were inserted into the P element vector Carnegie 30 (Mismer and Rubin, 1987) in the opposite orientation to the rosy$^+$ gene (Figure 1).

Analysis of transgenic flies

Injections were into a host strain carrying variants of the four LSP gene and a partial deletion of the rosy gene: LSP1$\alpha^F$; LSP1$\beta^o$; LSP1$\Gamma^o$LSP2$^+$ry$^{306}$, as described previously (Jowett 1985). Transformants were selected and mapped according to the scheme in Figure 2. Southern blotting identified those transformants which contained single insertions, and two transformants of each construct with insertions on chromosome II were selected for further study (Figure 3). These transgenic strains were homozygous viable and phenotypically normal.

The expression of the transgenes was analysed in a variety of ways. Firstly, since the LSP promoter is tissue and developmentally specific it was expected that the transgenes would be expressed solely in the larval fat body and only appear during the third instar. Mid-third instar larvae from each of the four transgenic strains were dissected and total RNA extracted from the fat body and carcass. Northern analysis of this RNA using the CYP2B1 gene, the lacZ gene construct and a portion of the LSP1$\alpha$ gene as probes confirmed that the lacZ and CYP2B1 transcripts were found in abundance in the fat bodies of the corresponding transgenic strains (Figure 4). The transcript from the $\alpha$CYP2B1 transgene was of the expected size although the alacZ gene appeared to produce two transcripts, one 100-200 bp larger than the other. Since

the Adh fragment in the αlacZ construct contains most of the proximal Adh promoter, this could be providing an alternative site for transcription initiation. The alpha gene fragment identified both the endogenous alpha transcript and the CYP2B1 transcript but not the very much less abundant lacZ transcript. The transgene transcripts had only 70bp of homology with the probe and so only weak hybridisation signals would be expected. Note also that the lacZ fragment used as probe contained this Adh fragment and therefore cross hybridised with the proximal and distal Adh transcripts found in the fat body and carcass of mid-third instar larvae. The presence of translation products of the transgenes was confirmed by staining the fat bodies with X-gal for the αlacZ transformants and immunologically for the αCYP2B1 larvae (Figure 5). Western blots of third instar larvae also showed that CYP2B1 was expressed only in the fat body of third instar larvae and that high levels of immunoreactive protein were detected in the endoplasmic reticulum containing microsomal fraction (Figure 6). The immunoreactive protein from transformants migrated as a doublet and it was the upper polypeptide that comigrated with the purified protein. Since the LSP1α promoter is very efficient and the amounts of protein are high, this may reflect incomplete post-translational modification of the protein in the larval fat body or partial degradation.

Larval microsomes from the strains expressing the P450 gene gave the characteristic carbon monoxide difference spectrum with a peak at 450nm, whereas microsomes from untransformed larvae gave no detectable P450 peak at the same protein concentration (data not shown). This suggested that not only was the protein likely to be biologically active, but it was also present at very much higher levels than the endogenous enzymes. In our experiments we were unable to detect endogenous P450 activity in larval microsomes; this presumably r fl cted the relatively low level of activity present in the control strain. In general, it has been

found that levels of total cytochrome P450 content are much lower in larvae than in adults and there are differences between strains (Hällström and Grafström, 1981; Zijlstra *et al*, 1984; Hällström *et al*, 1983).

In order to determine whether the expressed enzyme retained its normal substrate specificity microsomal preparations were assayed for their activities towards different model cytochrome P450 substrates, namely some resorufin analogues (Burke and Mayer, 1976). These substrates give a distinct pattern of activity with the different P450 isozymes.

**Table 1.**

| Source of enzyme activity of the form of purified cyt. P450. | Relative Activity* C2 | | C5 | | Benz |
|---|---|---|---|---|---|
| αlacZ microsomes | <0.02 | | <0.02 | | <0.02 |
| αCYP2B1 microsomes | <0.02 | < | 0.08 | < | 1.00 |
| P450IIB1 [a,b] | 0.05 | < | 4.91 | < | 9.10 |
| P450IIB1 [c] | 0.30 | < | 0.40 | < | 3.20 |
| P450IIB2 [a] | 0.03 | < | 0.04 | < | 0.16 |
| P450IIC6 [b] | 0.41 | > | 0.00 | < | 0.04 |
| P450IIC11 [b] | 0.46 | > | 0.04 | < | 0.30 |
| P450IA1 [b] | 28.0 | >> | 0.33 | << | 6.98 |
| P450IA1 [c] | 7.6 | >> | 0.06 | << | 3.3 |

**Table 1.** Comparison of the activities towards resorufin analogues of *Drosophila* microsomes from The αlacZ strain with those from the αCYP2B1 strain and with previously published data for the purified enzymes.

* Activities are expressed as nmol/min/mg protein for microsomal samples, and as nmol/min/nmol P450 for the purified proteins.

$C_2$ = ethoxyresorufin; $C_5$ = pentoxyresorufin; Benz = benzyloxyresorufin

[a] Data from Wolf *et al* (1988). [b] Data from Wolf *et al* (1986). [c] Data from Rodrigues *et al* (1987).

*Drosophila* expressing αCYP2B1 showed an activity which was higher towards 7-benzyloxyresorufin than 7-pentoxyresorufin, while no activity was detected towards 7-ethoxyresorufin, consistent with the relative activities published previously (Wolf *et al*, 1988; 1986; Rodrigues *et al*, 1987). Microsomes derived from αlacZ transformed larvae gave no detectable activities towards any of the three analogues. Interestingly, these data also demonstrate that the *Drosophila* P450-reductase can couple effectively with the mammalian P450 enzyme. When the same gene, CYP2B1, is expressed in *S. cerevisiae* it couples successfully with the yeast P450-reductase (Black *et al*, 1989).

**EXAMPLE 2: TRANSGENIC *DROSOPHILA*: GENOTOXICITY OF CYCLOPHOSPHAMIDE**

Having established that active P450 could be expressed in *Drosophila* larvae, we determined whether the enzyme had a biological effect *in vivo* using the SMART genotoxicity assay. The particular version of this test chosen was that described by Szabad (1986). This involves two complementary strains carrying genetic markers on the X, Y and third chromosomes. These strains are crossed to generate larvae heterozygous for each of the markers (Table 2). We have found that choosing the second chromosome for insertions of the transgenes allows them to be most easily introduced into the marker strains. In addition, two different chromosomal insertions for each construct were used so that the cross to generate the heterozygous larvae for the test would generate transheterozygous second chromosome insertions. This was an important feature of the experimental design as it eliminated any recessive effects caused by each particular insertion. Also, in generating the complementary parental strains, the integrity of each chromosome was maintained by ensuring that heterozygous chromosome combinations either involved

balancers or were in males (Figure 7). In this way changes in the genetic background were kept to a minimum. The materials and methods were as in Example 1.

Cyclophosphamide is a carcinogenic anticancer drug which is activated by cytochrome P450 *in vitro* to mutagenic derivatives (Nau *et al*, 1982). It is also activated in normal *Drosophila* by the endogenous enzyme(s) (Graf *et al*, 1983; Clements *et al*, 1984; 1990). However, since the levels of the transgenic product were so high, it was reasonable to expect that the transgenic P450-expressing strains would be hypersensitive to the drug. The lack of measurable endogenous P450 activity in larvae illustrates an important advantage of using an organism evolutionary distant from mammals in which to express the enzyme.

Injection of cyclophosphamide into third instar larvae

Larvae for treatment with cyclophosphamide were generated from by the following crosses:

(A) $\frac{fs(1)K10\ w\ \alpha lacZ\text{-}B3\ mwh\ se\ e}{B^{S}Y\qquad \alpha lacZ\text{-}B3\ mwh\ se\ e} \times \frac{y\ w^{co}\qquad \alpha lacZ\text{-}K2\ se\ flr^{3}}{y\ w^{co}\ \alpha lacZ\text{-}K2\ TM2,\ se}$

(B) $\frac{fs(1)K10\ w\ \alpha CYP2B1\text{-}D1\ mwh\ se\ e}{B^{S}Y\qquad \alpha CYP2B1\text{-}D1\ mwh\ se\ e} \times \frac{y\ w^{co}\qquad \alpha CYP2B1\text{-}C1\ se\ flr^{3}}{y\ w^{co}\ \alpha CYP2B1\text{-}C1\ TM2,\ se}$

αlacZ-B3, αlacZ-K2, αCYP2B1-D1 and αCYP2B1-C1 are independent single insertions of either the αlacZ or αCYP2B1 constructs on the second chromosome. The other symbols are described in Szabad (1986). 4h egg collections were made from the crosses above and the larvae reared until late third instar. The larvae were washed in Ringer, dried and etherized for 90sec. The anaesthetised larvae were injected with a 0.9% NaCl ± 10 mg/ml cyclophosphamide, using a drawn out glass-capillary connected to an air-

filled 20 ml glass syringe. Treated larvae were transferred to vials and allowed to develop into adults which were killed and their wings mounted on microscope slides in Faure's mountant (Ashburner, 1989). Wings were scored for multiple wing hair (mwh) and flare (flr) clones according to Graf *et al* (1984).

To ensure that all larvae were exposed to equivalent amounts they were injected with the drug in aqueous solution and allowed to develop to adults. Their wings were then scored for clones of marked cells. The results of administering cyclophosphamide to both the αlacZ and αCYPC2B1 transgenic larvae are given in Table 2a.

**Table 2a.**

| Strain and treatment | Number of wings | Type and number of spots (frequency in brackets) | | |
|---|---|---|---|---|
| | | Small | Large | Total |
| lacZ (CON) | 98 | 58 (0.59) | 21 (0.21) | 79 (0.81) |
| lacZ (CYC) | 140 | 635 (4.54) | 50 (0.36) | 685 (4.89) |
| P450 (CON) | 102 | 75 (0.74) | 14 (0.14) | 89 (0.87) |
| P450 (CYC) | 137 | 1165 (8.50) | 103 (0.75) | 1268 (9.26) |

**Tabl 2a.** SMART assay on the transgenic *Drosophila* larvae injected with cyclophosphamide.

The progeny from crosses A and B (see material and methods for Ex. 1) are referred to as lac? and P450 respectively. CON and CYC represent injection with saline and cyclophosphamide respectively.

Analysis of the wings for mosaics showed that in both strains cyclophosphamide caused a highly significant increase in the frequency of total clones in the wing:

**Tabl 2b.**

| TREATMENTS COMPARED | $N_c$ | $N_t$ | $n_c$ | $n_t$ | $f_c$ | $f_t$ | Chi-square |
|---|---|---|---|---|---|---|---|
| TOTAL WING SPOTS | | | | | | | |
| lacZ/CON v lacZ/CYC | 98 | 140 | 79 | 685 | 0.81 | 4.89 | 298.7** |
| P450/CON v P450/CYC | 102 | 137 | 89 | 1268 | 0.87 | 9.89 | 722.1** |
| lacZ/CON v P450/CON | 98 | 102 | 79 | 89 | 0.81 | 0.87 | 0.189 |
| lacZ/CYC v P450/CYC | 140 | 137 | 685 | 1268 | 4.89 | 9.26 | 186.3** |
| SMALL WING SPOTS | | | | | | | |
| lacZ/CON v lacZ/CYC | 98 | 140 | 58 | 635 | 0.59 | 4.54 | 306.6** |
| P450/CON v P450/CYC | 102 | 137 | 75 | 1165 | 0.74 | 8.50 | 678.6** |
| lacZ/CON v P450/CON | 98 | 102 | 58 | 75 | 0.59 | 0.74 | 1.339 |
| lacZ/CYC v P450/CYC | 140 | 137 | 635 | 1165 | 4.54 | 8.50 | 18.82** |
| LARGE WING SPOTS | | | | | | | |
| lacZ/CON v lacZ/CYC | 98 | 140 | 21 | 50 | 0.21 | 0.36 | 3.479 |
| P450/CON v P450/CYC | 102 | 137 | 14 | 103 | 0.14 | 0.75 | 43.86** |
| lacZ/CON v P450/CON | 98 | 102 | 21 | 14 | 0.21 | 0.14 | 1.283 |
| lacZ/CYC v P450/CYC | 140 | 137 | 50 | 103 | 0.36 | 0.75 | 18.82** |

** Significant at the P << 0.001 level.

**Table 2b.** Statistical analysis of the frequencies of clones in the two transformed strains. $N_c$ and $N_t$ are the number of wings in the control and test groups respectively. $n_c$ and $n_t$ are the number of spots in the control and testwings. $f_c$ and $f_t$ are the control and test group frequencies of spots per wing. Chi-squared was calculated as:

$$X^2 = \frac{(|n_c - P_o n| - 0.5)^2}{P_o n} + \frac{(|n_t - q_o n| - 0.5)^2}{q_o n}$$

where $P_o = N_c/(N_c + N_t)$ $q_o = N_t/(N_c + N_t)$ $n = n_c + n_t$

Background frequencies of mosaics in the two strains were low and no significant difference was found between them. Comparison of the frequency of mosaic spots between both strains treated with cyclophosphamide revealed a highly significant increase in senstivitiy towards the drug for the αCYP2B1 strain.

These data show that *Drosophila* expressing mammalian cytochrome P450 enzymes exhibit hypersensitivity towards cyclophosphamide-induced genotoxic effects. They also provide direct evidence that the enzyme metabolizes the compound to products which are mutagenic *in vivo*. It is important to note that the site of metabolic activation was the fat body whereas the target sites for the genotoxic effects were the wing imaginal discs. This clearly demonstrates the ability of the reactive metabolite to migrate from one cell type to another. These studies illustrate the potential of this system both for testing the role of specific rodent or human enzymes in the metabolism and activation/deactivation of chemical toxins and carcinogens and for studying the *in vivo* properties of chemical toxins and carcinogens themselves. The ability of reactive metabolites to migrate from their site of

activation to potential stem cell targets for example is a central issue in chemical carcinogenesis. The markers used in the SMART test allow detection of mosaics in different target tissues: the eye, wing and germline as well as nondisjunction in the male and female progeny of the F2 therefore allowing assessment of different genotoxic effects in different types of tissue (Szabad, 1986). This model can be applied to a wide range of enzymes involved in drug metabolism such as human cytochromes P450, glutathione S-transferases etc, as well as to important enzymes involved in protection against environmental chemicals, for example peroxidases, DNA repair enzymes and even membrane transport proteins such as P-glycoprotein. In addition, by crossing strains carrying different drug metabolizing enzymes, studies can be directed to dissect out the delicate balance between drug activation and detoxification. It will also allow complete metabolic cascades involving drug activation and detoxification pathways to be established. The power of *Drosophila* genetics make this just the starting point for the fine tuning of such metabolic systems.

*Drosophila* is used as an alternative to mammals for *in vivo* genotoxicity testing. There are marked differences in the ability of *Drosophila* to metabolize certain xenobiotics such as polycyclic aromatic hydrocarbons when compared with mammalian systems (Zijlstra *et al*, 1987). With the development of gene-targeted P element-induced mutation (Ballinger and Benzer, 1989; Kaiser and Goodwin, 1990), it is now possible to generate *Drosophila* strains in which endogenous genes for xenobiotic-metabolising enzymes have been replaced by human genes, providing a model system which can be tailored to resemble different human drug-metabolising combinations.

**EXAMPLE 3: TRANSGENIC YEAST EXPRESSING YEAST/RAT HYBRID P450 REDUCTASE**

Cytochrome P450 enzymes are a super genefamily of haemoproteins involved in a variety of biotransformation systems and are found in most organisms and in mammals in multiple forms. By contrast, the co-enzyme NADPH:cytochrome P450 reductase is only present in a single form in mammals and acts as a co-enzyme for all multiple forms of cyt. P450. Mammalian cyts. P450 cDNAs have been expressed with varying success in *S. cerevisiae* (eg Oeda *et al* 1985, Black *et al* 1989), while attempts to express mammalian P450 reductase have resulted in very low levels of expression (Murakami *et al* 1987). The expression of mammalian cyt. P450 systems in yeast allows study of individual enzyme metabolism potential and also allows the study of the interaction between cyt. P450 and P450 reductase. Overexpression of the endogenous *S. cerevisiae* P450 reductase has recently been reported and shown to improve the metabolic activity of mammalian cyts. P450 expressed in yeast (Murakami *et al* 1990). However, given that the identity of mammalian P450 reductases is usually over 90% and that the identity between rat and *S. cerevisiae* P450 reductases is only 35%, it is highly probable that this improvement in cyt. P450 activity will not always be possible by solely overexpressing the yeast enzyme. Here we report the construction of two yeast-rat P450 reductase fusions and their expression in *S. cerevisiae*.

**Bacterial and yeast strains:** *Escherichia coli* strain NM522 was used for cloning experiments. *S. cerevisiae* strain S150-2B (MATa,his3-Δ1,leu2-3,leu2-112,trp1-289,ura3-52,cir+) was used in all expression experiments.

**Media and Growth conditions:** Bacteria were cultivated in Luria-Bertani medium (Miller, 1972). Yeast transformed

with LEU2 selection plasmids were cultivated on minimal medium containing 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, 20μg per ml uracil, 2% agar and required amino acids (20μg per ml). Yeast transformed with TRP1 selection plasmids were cultivated on the same medium but with a supplement of 1% casamino acids (Difco) instead of single amino acids. Recombinant yeast strains were cultivated aerobically at 30°C in the above media without agar.

**Construction of P450 reductase fusion cDNAs and expression vectors.** The *S. cerevisiae* strong constitutive *ADH1* promoter was used throughout. This promoter has been successfully used for expression of mammalian cyts P450 in yeast previously (Oeda *et al* 1985). Hybrid cDNAs were constructed by adding an oligo encoding the *S. cerevisiae* P450 reductase n-terminus onto varying lengths of rat P450 reductase cDNA (Figure 8) and then the entire hybrid cDNA was then transferred on a single fragment into pAAH5 (Figure 9). A control plasmid, pFBY3, containing the complete rat reductase cDNA only was also constructed (Figure 9). The three different proteins produced by the constructs pFBY3, pFBY5 and pFBY6 and their molecular weights are illustrated in Figure 10.

**Expression of hybrid P450 reductase proteins.** Expression of the P450 reductase proteins in yeast was assessed using Western blotting (Figure 11) and also cytochrome c reductase activity (Table 3). In both cases it could be seen that the unmodified rat P450 reductase protein (pFBY3) was not expressed at detectable levels, while both the fusion proteins (pFBY5 and pFBY6) were produced at levels detectable by both Western blotting (Figure 11) and cytochrome c reductase activity (Table 3). Both the fusion proteins produced had the molecular weights predicted from their DNA sequences (Figures 10 and 11) and RNA of the correct molecular weight was detectable at similar levels

in transformants of all three constructs indicating that the lack of protein produced by pFBY3 was not caused by the promoter in any way. The lighter of the two P450 reductase fusions appears to be produced in lower quantities than the larger one suggesting that the problems in expressing mammalian P450 reductase in yeast are somehow caused by an unstable protein structure, although other explanations cannot be ruled out. The most stable of the three proteins (produced by pFBY6) contains both *S. cerevisiae* and the rat protein n-termini as well as the soluble moiety of the rat protein, indicating that while the presence of the yeast protein n-terminus contributes significantly to protein stability, possibly by allowing improved targeting into the membrane, the rat protein n-terminus also contributes to the general stability of the protein in some way. It is worth noting that these two n-terminal regions have very little identity compared to other regions of the proteins.

Thus the rat P450 reductase protein expression has been significantly stabilised in yeast by the addition to the protein of the yeast n-terminal 24 amino acids. This stabilisation appears to occur at the protein rather than the RNA level.

**EXAMPLE 4: TRANSGENIC YEAST CO-EXPRESSING HYBRID P450 REDUCTASE AND RAT P450**

As the protein produced by pFBY6 (see Example 3) was the most stable of the three, this fusion was selected for experiments in conjunction with cyt. P450 in yeast and to this end pFBY8 was constructed (Figure 9). This plasmid contains the cDNA for the hybrid P450 reductase protein as well as carrying a second *ADH1* promoter followed by a unique *Eco*RI site for cloning in cyt P450 cDNAs. A second advantage of this construct is that the TRP1 selection enables it to be grown on richer medium (see the Materials and Methods section of Example 3) which appears to improve

the yield of protein still further (Figure 11 and Table 3).

**Table 3.**

| PLASMID[a] | CYT.C REDUCTASE[b] ACTIVITY (UNITS) |
|---|---|
| pAAH5 | 0.04 |
| pFBY3 | 0.04 |
| pFBY5 | 0.09 |
| pFBY6 | 0.14 |
| pFBY8 | 0.20 |

[a] Plasmids and the recombinant proteins produced by expression in yeast strain S150-2B are described in Figures 8, 9 and 10.

[b] Cytochrome c reductase activity was performed on microsomal fractions (see legend to Figure 11). One unit is defined as 1μmole of cyt.c reduced/min/mg microsomal protein. Cyt.c reductase activity was measured using the method of Vermillion and Coon (1978) and protein was quantified using the method of Bradford (1976).

Rat cyt. P450 IIB1 cDNA has previously been expressed on a pMA56-based plasmid (Black *et al* 1989) so this cDNA was initially chosen for study in the pFBY8 based system (Figure 9) using p56/3a as a control plasmid and cloning the cDNA into pFBY8 to creat pFBY7 (Figure 9). While expression of both P450 reductase and cyt. P450 simultaneously appeared to have neither a stabilising or destabilising effect on either protein (Figures 12 and 13), the activity of the cyt. P450 IIB1 as measured by the Benzyloxyresorufin assay was seen to be significantly increased.

**EXAMPLE 5: TRANSGENIC YEAST EXPRESSING HYBRID P450 REDUCTASE AND HUMAN P450**

As the identity of human and rat P450 reductases is 93%, it was decided to further extend the expression system of Example 4 to a human cyt. P450 cDNA. Cyt. P450 IIA6 was selected for this (Miles *et al* 1990) and was cloned into pFBY8 to create pFBY9 (Figure 9). As this cDNA had not been previously expressed in yeast, a cyt. P450 only expression vector was also created by cloning the cDNA into pMA56 to make pFBY10 (Figure 9). Expression of this cyt. P450 was confirmed by Western blotting (Figure 13) and P450 reductase levels were also determined (Figure 12 and Table 4). Activity for this cyt. P450, as determined by the coumarin hydroxylase assay (Miles *et al*) was also found to be significantly increased when the P450 reductase fusion was present.

**Table 4.**

| PLASMID[a] | CYT.C REDUCTASE[b] ACTIVITY (UNITS) | BENZYLOXYRESORUFIN[c] ACTIVITY pmol/min/mg | COUMARIN HYDROXYLASE[d] ACTIVITY pmol/min/mg |
|---|---|---|---|
| p56/3a | 0.04 | 12 | - |
| pFBY7 | 0.20 | 50 | - |
| pFBY8 | 0.20 | nd | 0.3 |
| pFBY9 | 0.20 | - | 2.1 |
| pFBY10 | 0.04 | - | 1.3 |

[a] Plasmids were as described in Figure 9.

[b] Cyt.c reductase activity was measured as described in Table 3.

[c] Benzyloxyresorufin activity was determined for microsomal samples as described by Black *et al* (1989).

[d] Coumarin hydroxylase activity was determined for microsomal samples as described by Miles *et al* (1990).

Thus, simultaneous expression of the P450 reductase fusion with mammalian cyts. P450 proteins in yeast significantly raises the specific activity of the cyts. P450, without any visible stabilisation of the protein levels. These results indicate that activity of mammalian cyts.

P450 expressed in *S. cerevisiae* is significantly improved when P450 reductase is also overexpressed suggesting that the normal *S. cerevisiae* P450 reductase is deficient at least quantitatively and possibly qualitatively as well. This system will allow the improved expression of other mammalian cyts. P450 in yeast and also enable more detailed study of structural interactions of both cyt. P450 and P450 reductase.

**EXAMPLE 6: TRANSGENIC MICE**

With the intention of expressing mammalian (human) proteins (enzymes) in mouse skin, we have constructed a vector which contains the bovine keratin VI promoter. This promoter is homologous to the human keratin 10 and mouse keratin H59, all of which are almost exclusively active in the skin. This enables one to investigate the influence of an expressed enzyme on the toxicity of a chemical applied to a readily accessible and easily observable tissue (skin).

The bovine keratin (BK) promoter was kindly donated by José Jorcano but this or similar promoters are described in, for example, Ballieul *et al* (1990) (mouse suprabasal keratin 10 gene), Molt *et al* (1987) (human cytokeratins), Rieger *et al* (1985) (bovine type I cytokeratin), Blessing *et al* (1987) (four bovine type II cytokeratins) and Rothnagel *et al* (mouse K5, K14, K1 and K10 genes). The bovine keratin VI gene is equivalent to the human K10 gene, which is similar to the human K1 gene; the respective promoter from any of these gives skin-specific expression. The bovine keratin 4 promoter (equivalent to human K6) is specific for squamous epithelium, not only skin. Other skin-specific promoters include the tyrosinase promoter. On replacing the promoter's cap-site we cloned it into a vector designed by John Clark (Lathe *et al*, 1987) *Gene* **57**, 193-201), which enables the excision of intact linear inserts for the

production of transgenic mice. The cassette vector constructed (pMP172) and its method of construction are described in Figure 14.

Other promoters which are specific for the skin may also be used in a similar fashion, for example as discussed in Rothnagel *et al* (1990) *J. Invest. Dermatol.* **94(4)**, 573.

A procedure similar to that described by Murphy and Hanson (1987) (in *DNA Cloning*, Vol III, D M Glover (Ed), IRL Press, 1987), was used to generate transgenic mice which express the human alpha-class glutathione S-transferase $B_1$-$B_1$ isozyme in their skin.

Expression was confirmed by Western blotting and enzymatically by using the alpha-class-specific isomerase activity of GSTs with androsten-3,17-dione as substrate.

Pilot skin-painting experiments were conducted with heterozygous transgenic mice, investigating the protective role of GST-$B_1B_1$ from benz(a)pyrene(B(a)P)- and dimethylbenzanthracene(DMBA)-induced genetic damage. Using $^{32}$P-postlabelling, the levels of DNA-adducts in the skin and lungs between control and transgenic mice were compared. The results in Figure 16 show that the expressed enzyme produced a small decrease in B(a)P-induced adducts and a small increase in adducts by DMBA in the skin. In the lungs the level of genotoxic damage by DMBA was reduced by approximately 50% with a small increase in adducts from B(a)P treatment. Any effect in the lungs is not due to expression of the transgene in that tissue as there is no detectable level of expression as determined by Western blot and immunohistochemical analysis. These initial results show that GST-$B_1B_1$ may be involved in the biotransformation of DMBA and possibly B(a)P *in vivo*. The B(a)P result is at least consistent with previous reports, showing its metabolites to be poor substrates for the

alpha-class GSTs.

Homozygous mice, which express twice the level of protein in the skin as the heterozygous mice, may be used instead. Other genes which may be expressed in mouse skin and in the other transgenic hosts of the invention include human cytochrome P450 enzymes, UDP glucuronosyl transferases, epoxide hydrolases or any gene involved in xenobiotic metabolism, DNA repair genes or multidrug resistance genes.

The mouse models can then be used to test the involvement of specific gene expression on the toxicity or carcinogenicity of compounds of interest. By simple dermal application of the test chemical, one can then observe the toxic responses on the skin of transgenic versus non-transgenic mice. This system can be used to establish the potential risk of industrial chemicals or drugs in development to man and can also be applied to determine which chemicals are substrates for the different human or other drug metabolic enzymes expressed in the skin. Such systems will also allow pathways important for the activation or deactivation of chemical carcinogens to be evaluated. Other models derived from the original transgenic lines can be obtained by crossing homozygous lines with mice which: (1) are transgenic for a second metabolic enzyme, allowing the investigation of a combination of metabolic pathways in chemical toxicity and carcinogenicity, and (2) carry mutations or gene deletions of interest which may make the test more sensitive or provide better analytical end points.

Creation of a Transgenic Mouse

Single cell mouse embryos are harvested from female mice that are impregnated the evening before. The embryos are treated with hyaluronidase and briefly cultured in M16 medium. The mbryos are transferred to M2 medium on a

microscop glass depression slide. The embryos are observed with a 40X objective and a 10X eyepiece using a Nikon Diaphot microscope equipped with Hoffman optics. The embryos are held in place with a holding pipette that has been rounded with a microforge. The positions of both the holding pipettes and the injection pipettes are controlled with micromanipulators. DNA as described below is loaded in the injection pipette at a concentration of 1 to 10 micrograms per milliliter. Approximately one picoliter, as judged by a refractile change of the pronucleus, of DNA solution is injected into the male pronucleus.

After DNA injection the embryos are transferred to M16 medium and incubated at 37°C in a 5% $CO_2$ atmosphere for one to two hours. Lysed embryos are discarded and embryos that appeared normal are transferred to one of the fallopian tubes of pseudopregnant foster mothers. The transfers are performed under a dissecting microscope using general anaesthesia (avertin).

After birth, newborn mice are kept with their foster mothers for 2 weeks, at which point they are then weaned and screened for DNA integration. A 2 cm portion of the tail is removed and homogenized in 2 ml of a solution of 0.1 M NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA for short duration, but long enough to disrupt cell and nuclear membranes. The homogenized tissue is treated with 50 U/ml RNaseA and 0.1% SDS for 15 minutes at 37°C. The mixture is exposed to Proteinase K digestion for 3 hours at 55°C followed by three extractions with phenol/chloroform. DNA is then precipitated by the addition of ethanol. After resuspending the precipitated DNA in 10 mM Tris pH 8.0, 0.5 mM EDTA, some of it is digested with *Bam*HI endonuclease and electrophoresed through an 0.8% agarose gel. The DNA is denatured by soaking the gel in 1.5 M NaCl, 0.5 M NaOH for one hour and then neutralizing the DNA by soaking it in 1.5 M NaCl, 0.5 M Tris, pH 7.4 for 30 minutes. The gel is then

soaked in 10X SSC for one hour. The DNA is then transferred from the gel onto a nitrocellulose filter by the method of Southern, as described in Maniatis, T. *et al.*, *Molecular Cloning, A Laboratory Manual*, pp 109-110, 383-389 (Cold Spring Harbor, New York 1982).

The filter with transferred DNA is hybridized overnight with $^{32}P$ labelled lambda DNA prepared, according to standard procedures, by the method of nick translation (Maniatis, *supra*). Following this overnight hybridization, the filter is washed in 0.1 x SSC, 0.1% SDS at 50°C and Kodak XAR film is exposed to it in order to identify lambda DNA present within the mouse genome. Lambda DNA, used as standards, that has been electrophoresed alongside the mouse genomic DNA is compared in intensity to the transgenic mouse DNA hybridized to the $^{32}P$ labelled lambda DNA to estimate copy number. Numerous transgenic animals may be produced and identified by this technique and most of them transmit the integrated DNA to their offspring, demonstrating germ line integration.

The inserted DNA can, theoretically, contain any number or variety of genes. In the prototype described herein, an *E. coli* bacteriophage lambda genome has been engineered to carry a $\beta$-galactosidase test DNA sequence. The genotype of the modified lambda genome L2B is lac5 delta (shindill lambda 2-3°) srl lambda 3-5° c1857 xXhl lambda 1° sScll lambda 4°. Before injecting it into mouse embryos, this lambda DNA is diluted to a concentration of 10 micrograms per millilitre and the cos ends are annealed and ligated under conditions predominantly forming circular lambda phage monomers (Maniatis, *supra*).

Newborn mice are tested for the presence of the test DNA sequence by the tail-blotting procedure (Hogan *et al.*, *Manipulating the Mouse Embryo: A Laboratory Manual* pp 174-183, Cold

Spring Harbor Laboratory, 1986). Several of the newborns are found to carry the test DNA sequence in DNA isolated from their tails. Eight weeks after birth these transgenic mice are mated and their progeny are examined for the test DNA sequence. Approximately 50% of the resulting offspring carried the test DNA sequence, demonstrating that the original transgenic mice carried the test DNA sequence in their germ line and that this sequence is inherited normally. While transgenic lines having approximately one copy of the test DNA sequence per cell can be obtained, lines having at least about 5-10 copies per cell are preferred.

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**CLAIMS**

1. A transgenic cellular organism useful in an assay for determining the metabolism of a compound, the organism comprising in the genome of its cell or at least one of its cells a coding sequence for expressing a polypeptide having the function of a naturally-occurring protein which is involved in the alteration of the mutagenicity or toxicity of a compound under the regulatory control of a suitable promoter, the combination of the coding sequence and the promoter not normally being found in the said cell of the said organism.

2. An organism according to Claim 1 which is a *Drosophila* fly, or a larva or egg thereof.

3. An organism according to Claim 2 wherein the said promoter provides expression in the fat body of the third instar larva.

4. An organism according to Claim 3 wherein the promoter is the *Drosophila LSP1α* promoter.

5. An organism according to Claim 1 wherein the organism is a mammal, the said cells which have the said coding sequence include skin cells and the said promoter is such as to provide expression of the coding sequence in the skin cells.

6. An organism according to Claim 5 wherein the promoter is the promoter from a mammalian keratin gene.

7. An organism according to Claim 5 or 6 which is a rodent.

8. An organism according to any on of Claims 5 to 7 wherein the cells having the said coding sequence also have an expressible coding sequence for a polypeptide having the enzymatic activity of a (preferably mammalian) glutathione S-transferase.

9. An organism according to Claim 8 wherein the glutathione S-transferase is mammalian.

10. An organism according to Claim 1 which is a yeast.

11. An organism according to Claim 10 wherein the said polypeptide comprises a region adapted to bind yeast membranes such that the polypeptide may be bound to such membranes without abolishing the said enzymatic activity.

12. An organism according to Claim 11 wherein the membranes are endoplasmic reticulum.

13. An organism according to Claim 10, 11 or 12 wherein the yeast also comprises means to express a polypeptide having the enzymatic activity of a non-yeast NADPH:cytochrome P450 reductase or means to express an increased level of a yeast NADPH:cytochrome P450 reductase.

14. An organism according to any one of the preceding claims wherein the polypeptide has the function of the mdr transport protein, a glutathione S-transferase or an enzyme from the superfamily of P450 cytochrome dependent enzymes.

15. A fusion protein comprising a first region providing the activity of a naturally-occurring protein from a first organism which protein is involved in the alteration of the mutagenicity or toxicity of a compound and a second region adapted to bind to cell membranes, the second region being homologous to a cell-binding region of a protein in an organism other than the first organism.

16. A fusion protein according to Claim 15 wherein the first organism is a mammal and the said naturally-occurring protein is the mdr transport protein, a glutathione S-transferase, P450 reductase or an enzyme of the superfamily of P450 cytochrome dependent enzymes.

17. A fusion protein according to Claim 15 or 16 wherein the second region is adapted to bind to yeast cell membranes.

18. A fusion protein according to Claim 15, 16 or 17 which comprises the sequence $H_2N-R^1-R^2-R^3-COOH$ wherein $R^1$ is homologous to the n N-terminal amino acids of *Saccharomyces cerevisiae* P450 reductase where n is 10-30, $R^2$ is homologous to the n′ N-terminal amino acids of human P450 reductase where n′ is 10-56, and $R^3$ provides the enzymatic function of human P450 reductase.

19. A fusion protein comprising a first region having the enzymatic function of a mammalian P450 enzyme and a second region having the enzymatic function of a P450 reductase, preferably a mammalian P450 reductase.

20. A method of preparing a transgenic cellular organism according to any one of Claims 1 to 14, comprising introducing into a cell of a cellular organism a heritable coding sequence and regulatory sequences therefor adapted to express, in the said cell or in progeny thereof, a polypeptide which is heterologous to the organism and which is involved in the mutagenicity or toxicity of a compound.

21. A method according to Claim 20 wherein the polypeptide increases or decreases the mutagenicity or toxicity of a compound.

22. A method of determining the toxicity or mutagenicity of a compound, the method comprising exposing an organism according to any one of Claims 1 to 14 to the compound and identifying mutations or toxic effects.

23. A method according to Claim 22 wherein the method also comprises exposing a second organism, which second organism is identical to the said organism but which lacks the function of the said polypeptide, to the compound and comparing the mutations or toxic effects of the compound on the said two organisms.

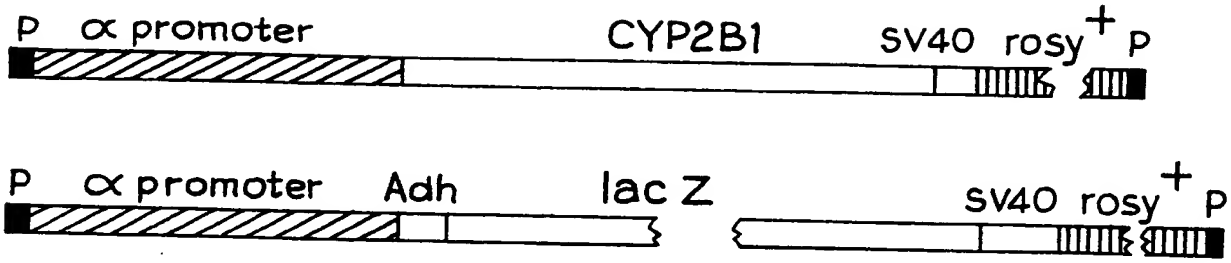


Fig. 1

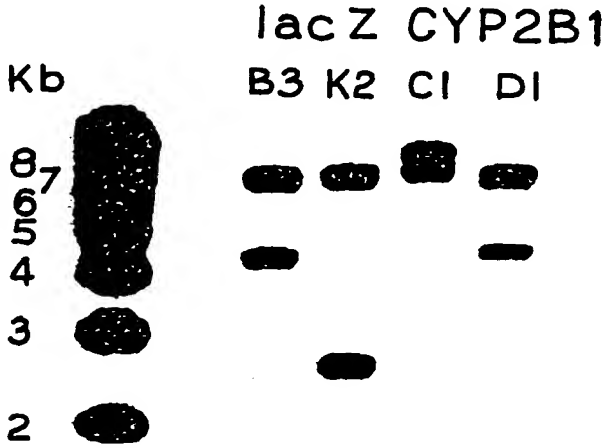


Fig. 3

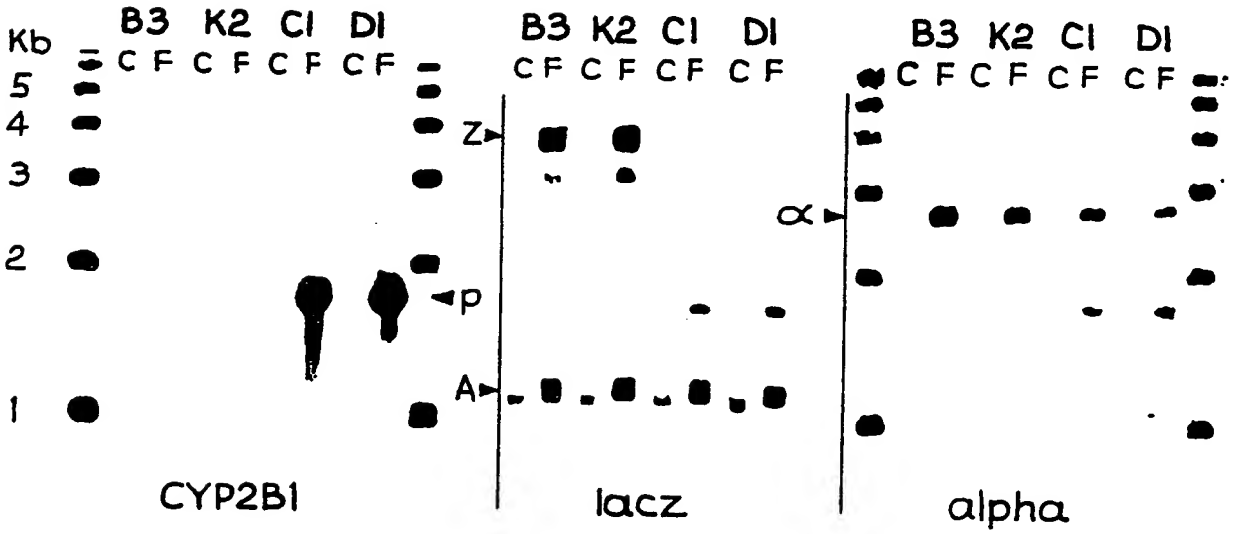


Fig. 4

SELECTION OF TRANSFORMANTS

Host Strain

$$\frac{\alpha^F\ \beta^\circ\ \Gamma^\circ\ 2^S\ ry}{\alpha^F\ \beta^\circ\ \Gamma^\circ\ 2^S\ ry} \times \frac{\alpha^F\ \beta^\circ\ \Gamma^\circ\ 2^S\ ry}{\alpha^F\ \beta^\circ\ \Gamma^\circ\ 2^S\ ry}$$

Inject embryos with Ppi25.7wc & P[ry$^+$]

Ppi25.7wc helper P element to supply transposase P[ry$^+$] P element construct carrying ry$^+$ gene and transgene construct

G0 Mate all surviving adults with host strain.

G1 Select transformed ry$^+$ flies x $\frac{\alpha^F}{\alpha^F/Y}\ \frac{\beta^\circ}{SM1}\ \frac{\Gamma^\circ\ 2^S\ ry}{TM3,\ Sb\ ry^{RK}}$

After mating extract DNA from ry$^+$ parents for genomic Southern analysis.

Select male, ry$^+$, Cy, Sb.
* denotes a possible P element insertion

G2 $\frac{\alpha^F*}{Y}\ \frac{\beta^\circ*}{SM1}\ \frac{\Gamma^\circ\ 2^S\ ry*}{TM3,\ Sb\ ry^{RK}}$ x $\frac{\alpha^F\ \beta^\circ\ \Gamma^\circ\ 2^S\ ry}{\alpha^F\ \beta^\circ\ \Gamma^\circ\ 2^S\ ry}$

Select male, ry$^+$, Sb

G3 $\frac{\alpha^F}{Y}\ \frac{\beta^\circ\ P[ry^+]}{\beta^\circ}\ \frac{\Gamma^\circ\ 2^S\ ry}{TM3,\ Sb\ ry^{RK}}$ x $\frac{\alpha^F\ \beta^\circ}{\alpha^F\ SM1}\ \frac{\Gamma^\circ\ 2^S\ ry}{TM3,\ Sb\ ry^{RK}}$

G4 $\frac{\alpha^F\ \beta^\circ\ P[ry^+]}{\alpha^F\ SM1}\ \frac{\Gamma^\circ\ 2^S\ ry}{TM3,\ Sb\ ry^{RK}}$ x $\frac{\alpha^F}{Y}\ \frac{\beta^\circ\ P[ry^+]}{SM1}\ \frac{\Gamma^\circ\ 2^S\ ry}{TM3,\ Sb\ ry^{RK}}$

G5 $\frac{\alpha^F\ \beta^\circ\ P[ry^+]}{\alpha^F\ \beta^\circ\ P[ry^+]}\ \frac{\Gamma^\circ\ 2^S\ ry}{TM3,\ Sb\ ry^{RK}}$ x $\frac{\alpha^F}{Y}\ \frac{\beta^\circ\ P[ry^+]}{\beta^\circ\ P[ry^+]}\ \frac{\Gamma^\circ\ 2^S\ ry}{TM3,\ Sb\ ry^{RK}}$

**FIGURE 2**

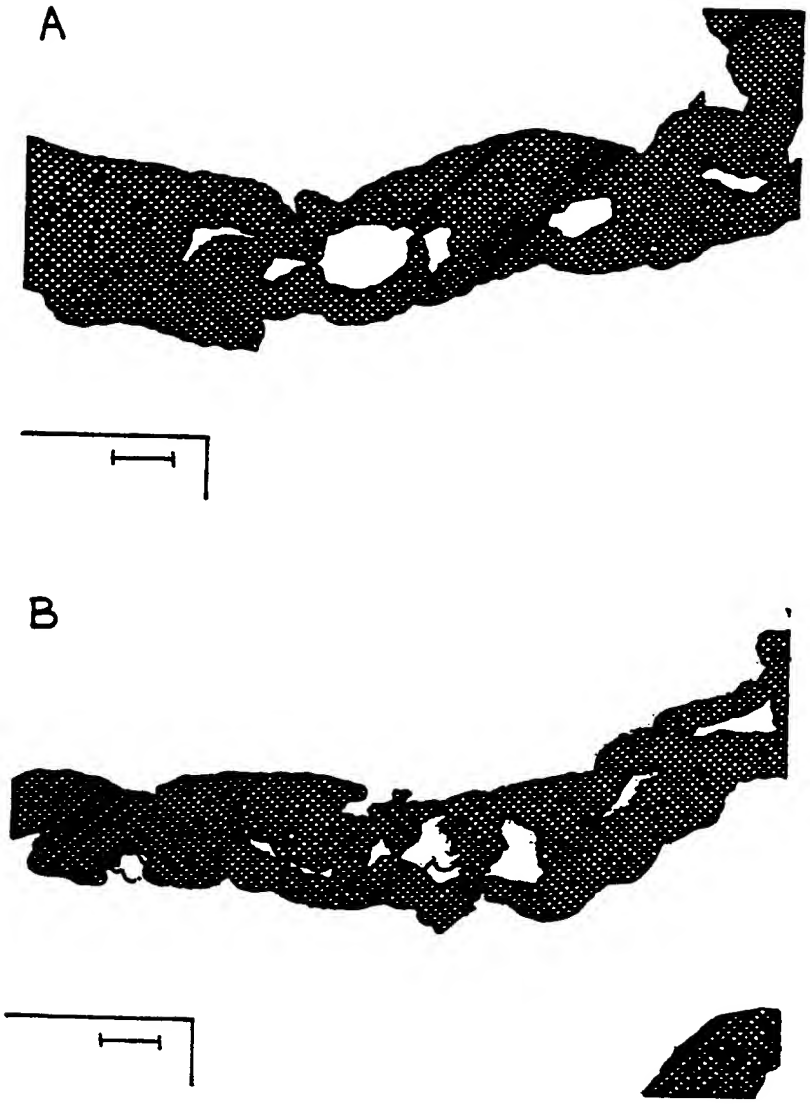


Fig. 5

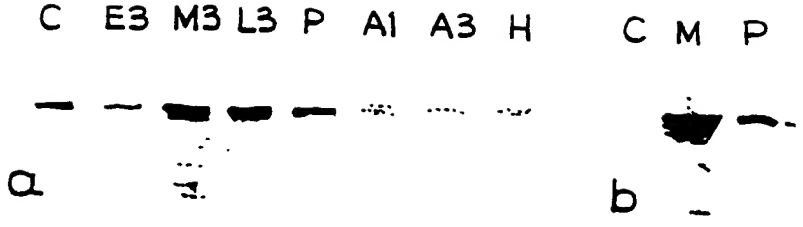


Fig. 6

## Breeding Scheme A

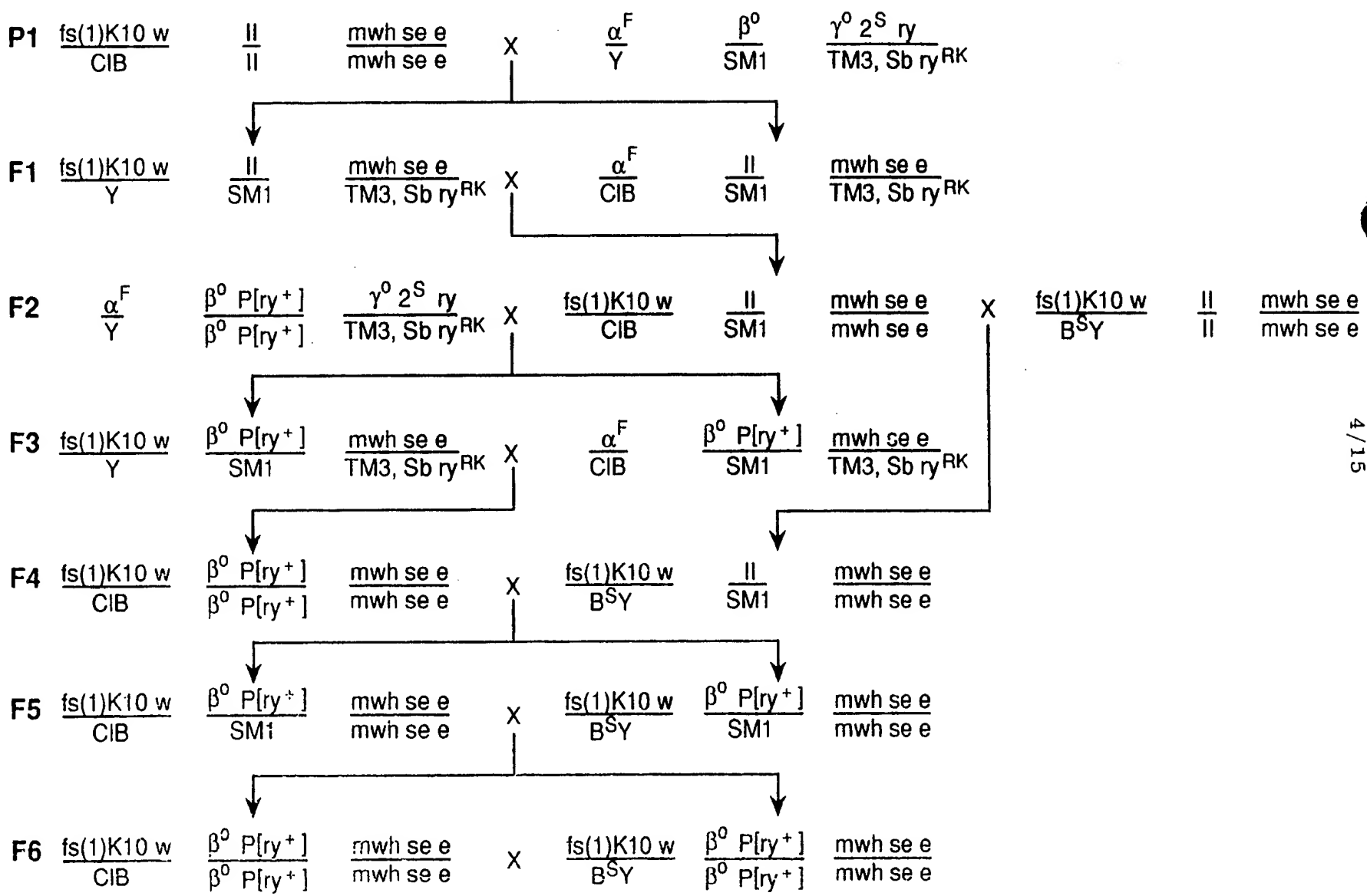


FIGURE 7 (START)

## Breeding Scheme B

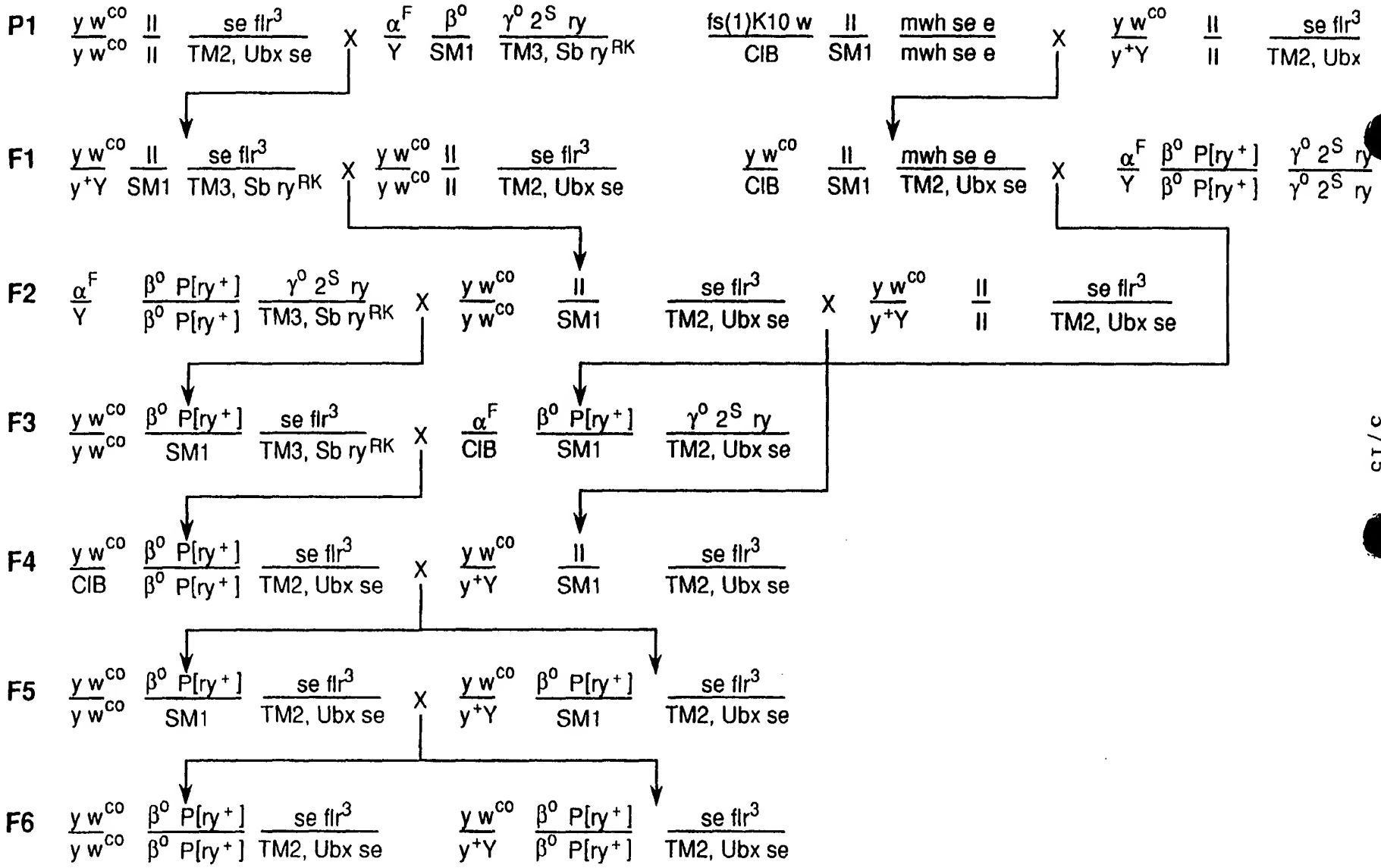


FIGURE 7 (END)

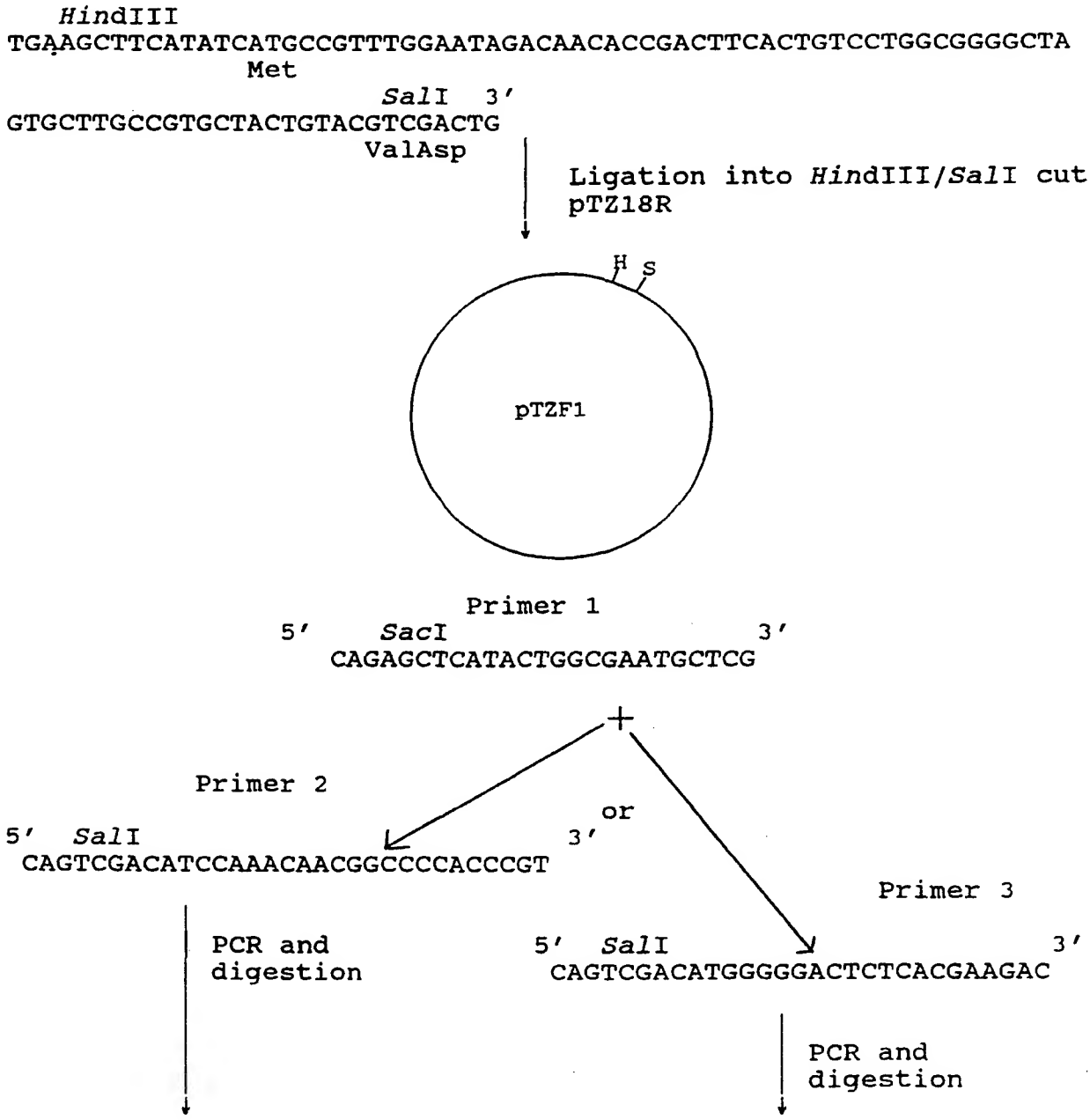


FIGURE 8 [START]

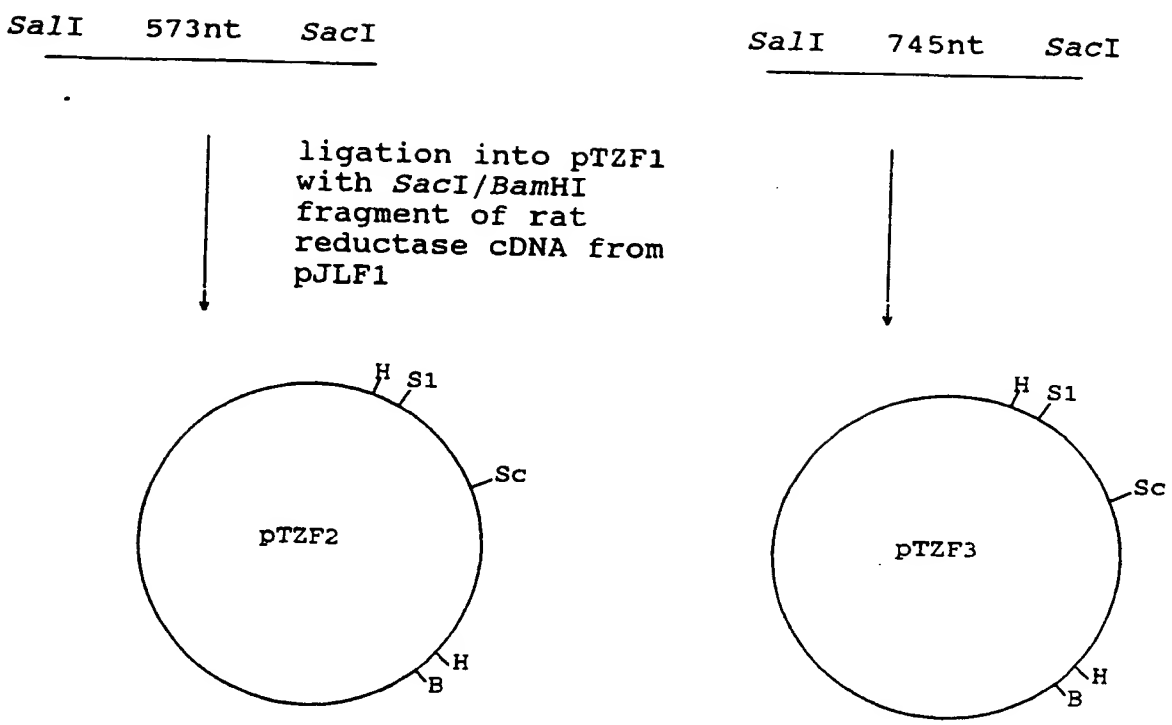
SalI 573nt SacI
SalI 745nt SacI
ligation into pTZF1 with SacI/BamHI fragment of rat reductase cDNA from pJLF1
H
S1
Sc
pTZF2
H
B
H
S1
Sc
pTZF3
H
B

FIGURE 8 [END]

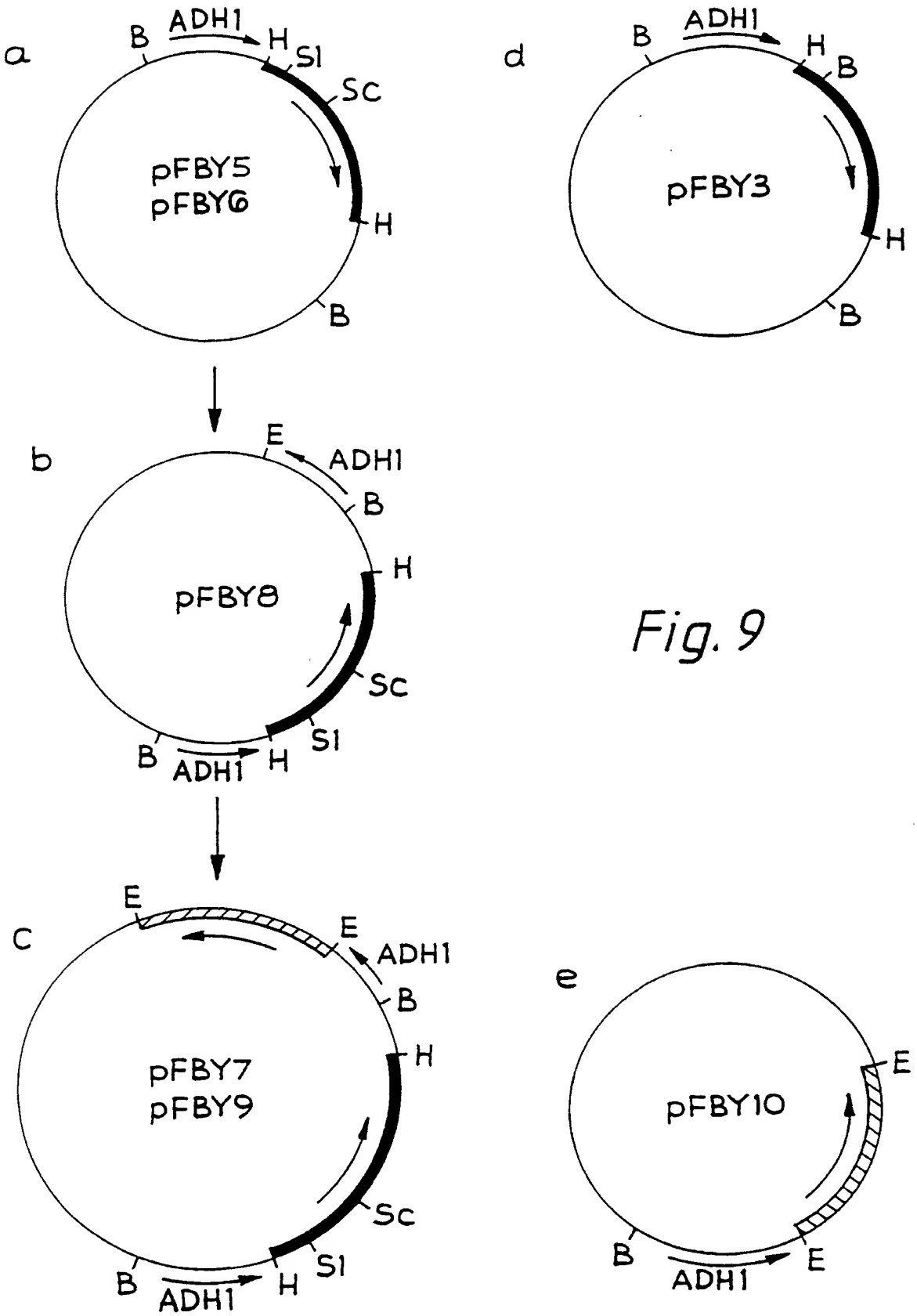


Fig. 9

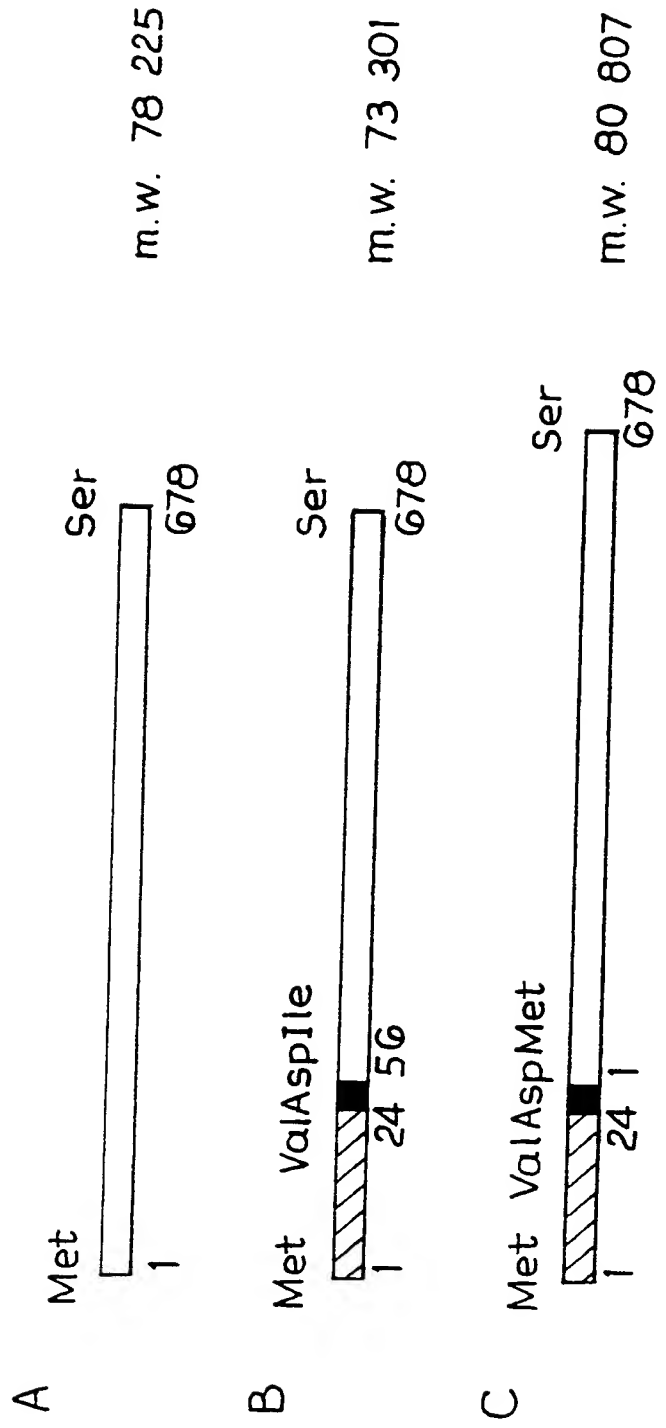
m.w. 78 225
m.w. 73 301
m.w. 80 807
Met
Ser
678
1
A
Met ValAspIle
24 56
B
Met ValAspMet
24 1
C

Fig. 10

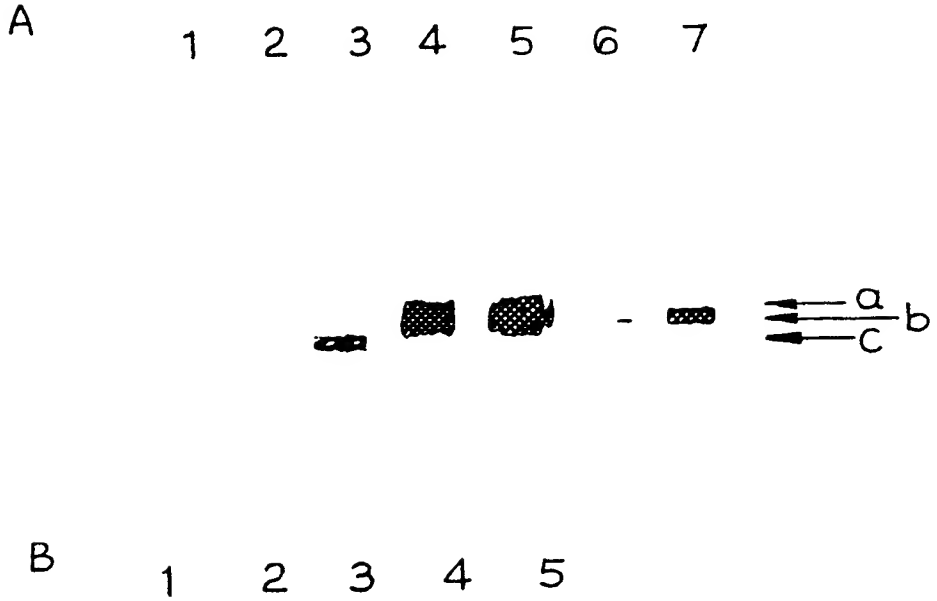


Fig. 11

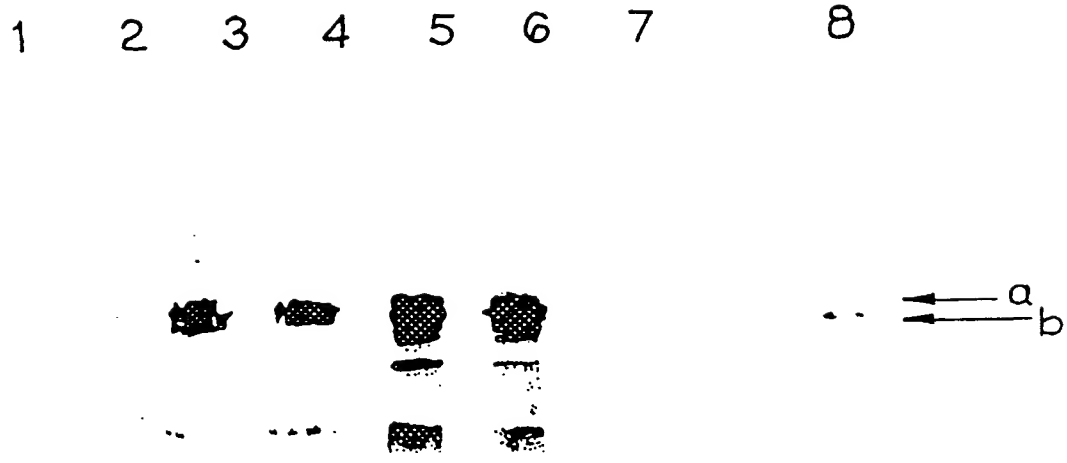


Fig. 12

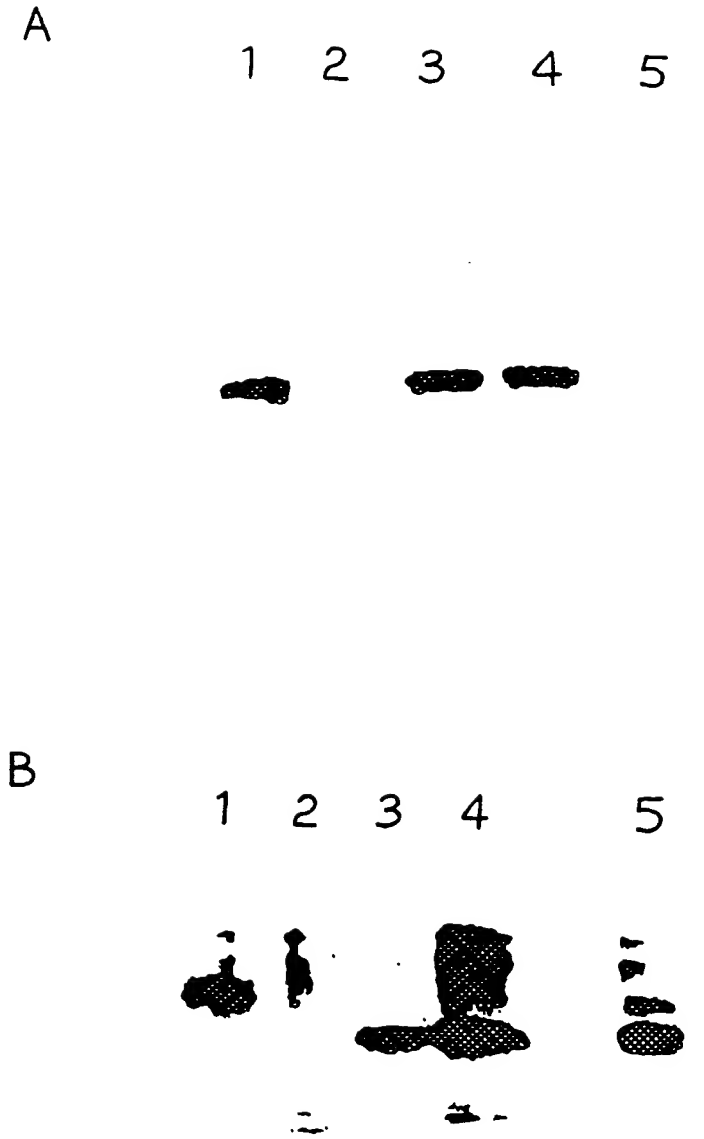


Fig. 13



SUBSTITUTE SHEET

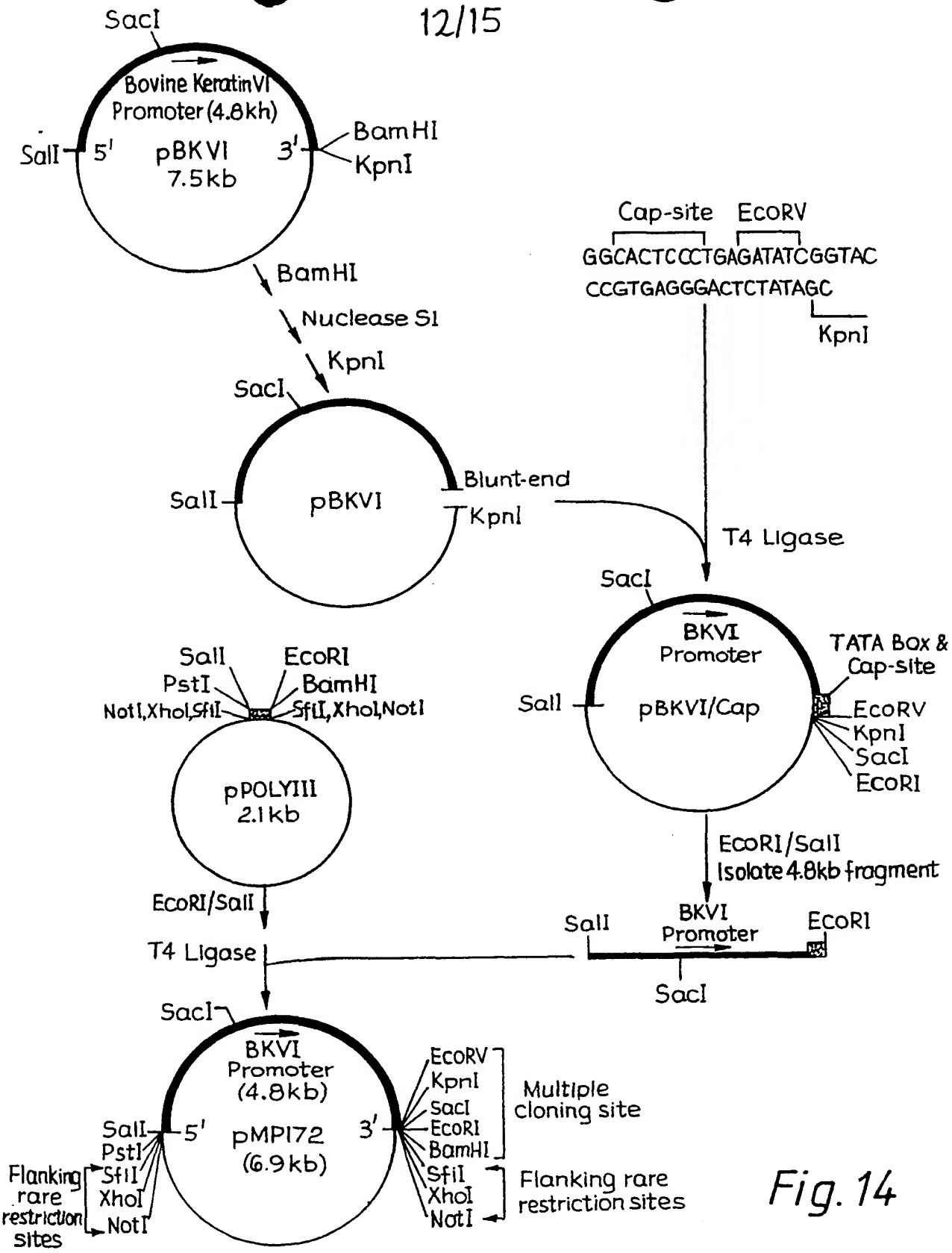


Fig. 14

Construction of Bovine Keratin VI expression vector for skin-specific expression in transgenic mice.



WO 0214817A1 | >

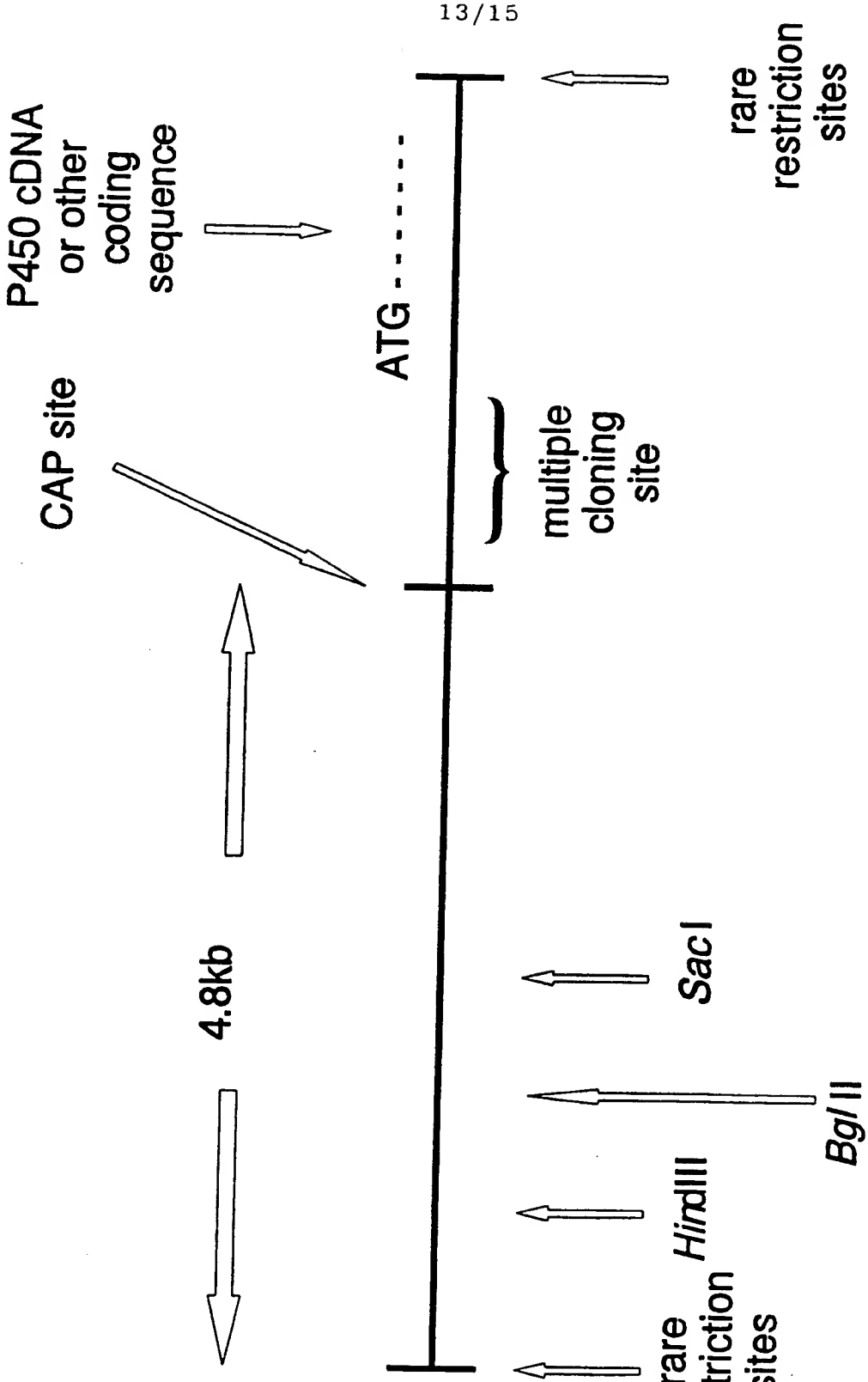
P450 cDNA or other coding sequence
CAP site
ATG - - - - - - -
rare restriction sites
multiple cloning site
4.8kb
SacI
BglII
HindIII
rare restriction sites

Figure 15

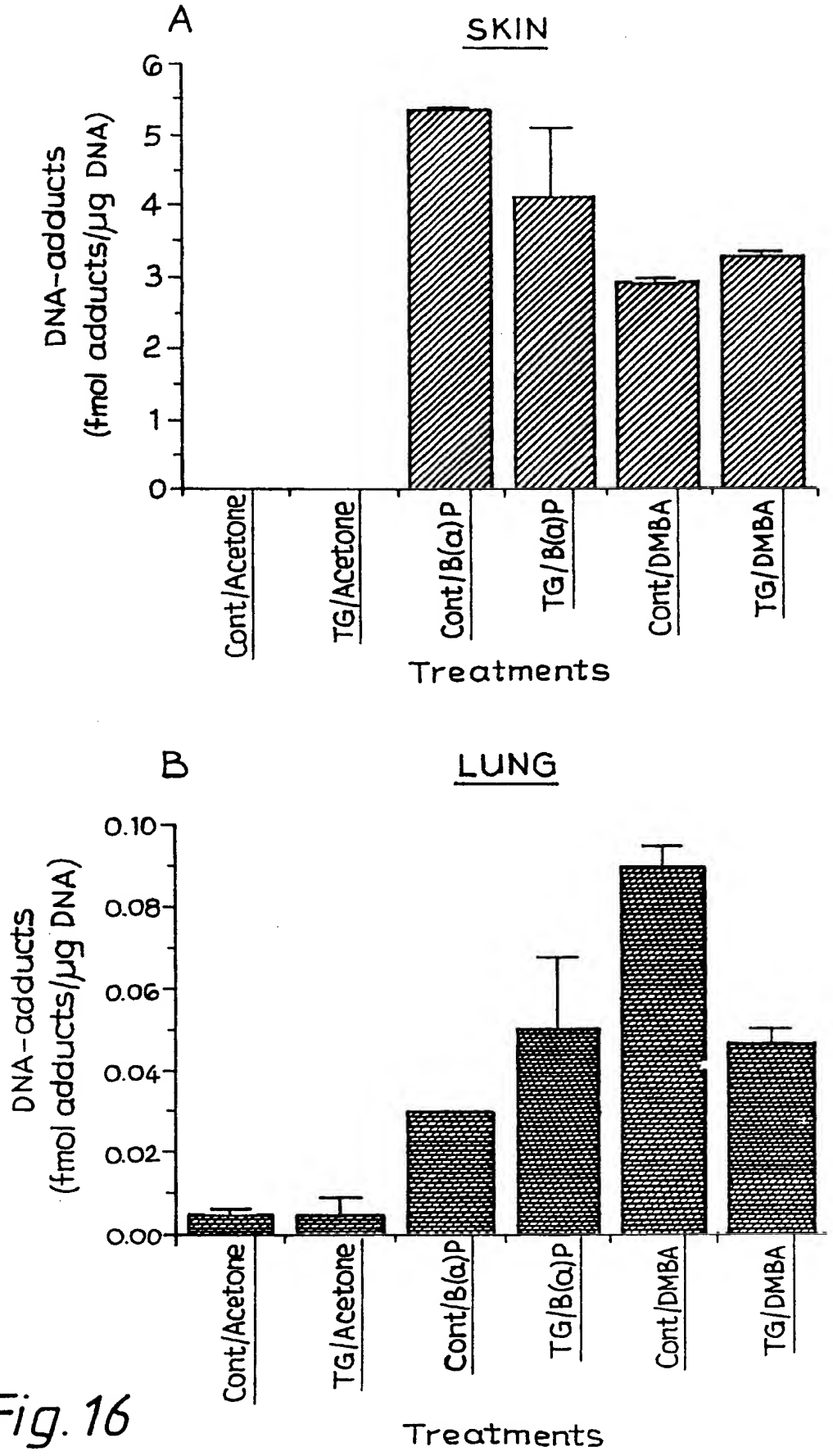


Fig. 16

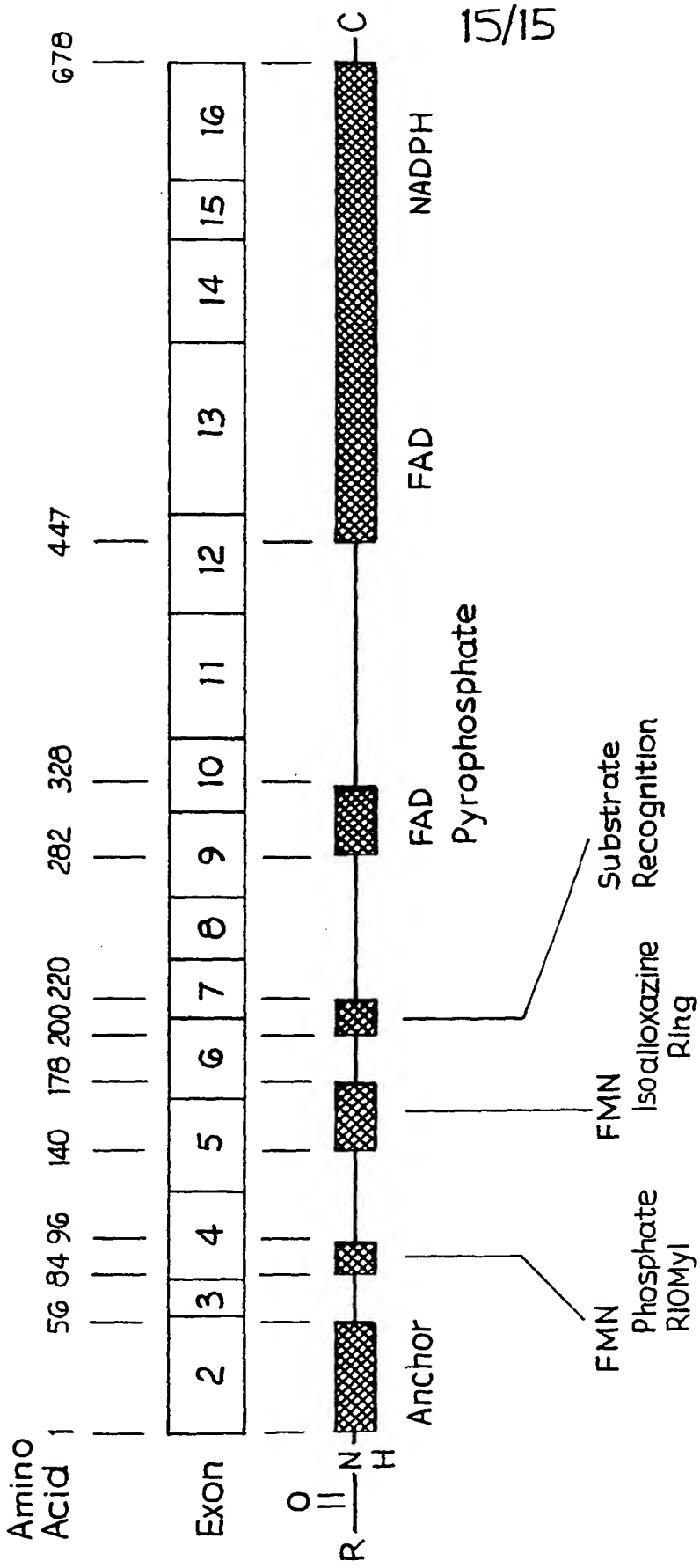


Fig. 17

# INTERNATIONAL SEARCH REPORT

International Application PCT/GB 92/00274

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/00; C12N9/02; C12N15/62; A01K67/027
C12N1/19; C12N9/10; C12N15/12; C12N15/53

## II. FIELDS SEARCHED

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|---|---|---|
| P,X | EMBO JOURNAL.<br>vol. 10, no. 5, May 1991, EYNSHAM, OXFORD GB<br>pages 1075 - 1081;<br>JOWETT, T ET AL.: 'Mammalian genes expressed in Drodophila : a transgenic model for the study of mechanisms of chemical mutagenesis and metabolism'<br>see the whole document | 1-4,14,<br>20,21 |
| | --- | |
| X | JOURNAL OF BIOTECHNOLOGY.<br>vol. 9, no. 4, March 1989, AMSTERDAM NL<br>pages 255 - 272;<br>ZURBRIGGEN,B. ET AL.: 'Controlled expression of heterologous cytochrome P450e cDNA in Saccharomyces cerevisiae. I Construction and expression of a complete rat cytochrome P450e cDNA' | 1,10,14,<br>20,21 |
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| X | DNA CELL BIOLOGY<br>vol. 9, no. 8, 1990,<br>pages 603 - 614;<br>SAKAKI, T. ET AL.: 'Expression of bovine cytochrome P450c21 and its fused enzymes with yeast NADPH-cytochrome P450 reductase in Saccharomyces cerevisiae'<br>& CHEMICAL ABSTRACTS, vol. 114,<br>1991, Columbus, Ohio, US;<br>abstract no. 18819F,<br>page 202 ;column 1 ;<br>see abstract | 1,10,14, 19 |
| X | EP,A,0 404 183 (OCCIDENTAL CHEMICAL CORPORATION) 27 December 1990<br>see the whole document | 1,20,21 |
| Y | WO,A,9 001 545 (ARCH DEVELOPMENT CORPORATION) 22 February 1990<br>see the whole document | 1,20,21 |
| X | WO,A,9 002 551 (BIOSOURCE GENETICS CORPORATION) 22 March 1990<br>see claims; examples | 1,5,20, 21,22 |
| P,X | WO,A,9 103 561 (E.I. DU PONT DE NEMOURS AND COMPANY) 21 March 1991<br>see the whole document | 1,14 |
| X | WO,A,9 004 632 (of commerce THE UNITED STATES OF AMERICA, THE SECRETARY, DEPARTMENT OF COMMERCE) 3 May 1990<br>see the whole document | 1,5,7, 14,20,21 |
| Y | EP,A,0 370 813 (TRANSGENIC SCIENCE) 30 May 1990<br>see the whole document | 20,21,22 |

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SA 56418

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| EP-A-0404183 | 27-12-90 | CA-A- | 2018884 | 22-12-90 |
| | | JP-A- | 3080089 | 04-04-91 |
| WO-A-9001545 | 22-02-90 | None | | |
| WO-A-9002551 | 22-03-90 | AU-A- | 4327089 | 02-04-90 |
| | | EP-A- | 0434750 | 03-07-91 |
| | | JP-T- | 4500672 | 06-02-92 |
| WO-A-9103561 | 21-03-91 | AU-A- | 6272990 | 08-04-91 |
| WO-A-9004632 | 03-05-90 | AU-A- | 4494589 | 14-05-90 |
| | | CA-A- | 2001128 | 21-04-90 |
| | | EP-A- | 0451157 | 16-10-91 |
| EP-A-0370813 | 30-05-90 | CA-A- | 2003415 | 25-05-90 |
| | | JP-A- | 2145200 | 04-06-90 |

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A2

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9615498.4 24 July 1996 (24.07.96) GB

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*Without international search report and to be republished upon receipt of that report.*

(54) Title: DROSOPHILA MELANOGASTER P70 S6 KINASE

(57) Abstract

The invention provides *Drosophila melanogaster*p70$^{S6K}$, as well as nucleic acids encoding this kinase. The sequence of *Drosophila* p70$^{S6K}$ and the gene encoding it are represented in SEQ ID NO. 2 and 1 respectively. The invention moreover provides mutated forms of *Drosophila* p70$^{S6K}$, including constitutively active and dominant negative forms thereof, which are useful in the study of p70$^{S6K}$ activity. Furthermore, the invention provides expression systems which produce *Drosophila* p70$^{S6K}$ in *Drosophila* and other organisms, and in particular systems in which expression of *Drosophila* p70$^{S6K}$ has been modulated so as to facilitate the study of its activity.

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# DROSOPHILA MELANOGASTER P70 S6 KINASE

The present invention relates to a protein kinase enzyme, specifically to p70 S6 kinase ($p70^{S6K}$) isolated from *Drosophila melanogaster.*

In the signal transduction pathway mediating the multiple phosphorylation of 40S ribosomal protein S6 in response to mitogens and oncogenes, the most proximal signalling components to S6 are $p70^{S6K}$ and $p85^{S6K}$ (Ferrari, S. & Thomas, G. (1994) CRC Crit. Rev. Biochem. Mol. Biol. 29, 385-413; Jefferies, H.B.J. & Thomas, G. (1995) in Translational Control, eds. Hershey, J.W.B., Mathews, M.B. & Sonenberg, N., Cold Spring Harbor Press, Cold Spring Harbor). Both of these kinase isoforms are generated from a common mRNA transcript through the use of alternative initiation translation start sites, residing within 23 codons of one another. The additional 23 amino acid extension at the amino terminus of $p85^{S6K}$ contains a nuclear localisation sequence, which constitutively targets this isoform to the nucleus, whereas $p70^{S6K}$ appears to be exclusively cytoplasmic. Consistent with the localisation of S6 in both compartments of the cell, the available data suggest that both kinase isoforms are regulated in a closely co-ordinated fashion. Little is known regarding the functional role of $p85^{S6K}$ and phosphorylated S6 in the nucleus, however recent studies employing the immunosuppressant rapamycin have led to the hypothesis that $p70^{S6K}$, through increased S6 phosphorylation, is involved in the selective translational upregulation of a family of mRNAs characterised by a oligopyrimidine tract at their 5' transcriptional start sites. Although these mRNAs, termed 5'TOP, comprise a small family, they can represent up to 20-30% of the cells mRNA transcripts, and in most cases code for essential gene products of the translational apparatus, such as ribosomal proteins.

The link between mitogen-induced $p70^{S6K}$ activation and the translational upregulation of 5'TOP mRNAs is based on the selective effects of the rapamycin-FKBP12 gain-of-function inhibitory complex on both responses. Activation of $p70^{S6K}$ is associated with the phosphorylation of the enzyme at multiple residues, which exhibit two distinct phosphorylation motifs. The sites initially identified are flanked by a proline in the +1 position (Ferrari, S., *et al.* (1992) Proc. Natl. Acad. Sci. USA 89, 7282-7285) and, with the exception of S411, are rapamycin resistant. The second set of sites is flanked in the +1 and -1 positions by large aromatic residues, and exhibit rapamycin sensitivity (Pearson, R.B., *et al.* (1995) EMBO J. 14, 5279-5287). Of these latter sites the principal target of

rapamycin-induced p70$^{S6K}$ inactivation and dephosphorylation is T389 in the linker region, that couples the catalytic and autoinhibitory domains. Conversion of this site to an acidic residue confers rapamycin resistance and constitutive activity on the kinase. Truncation mutants of p70$^{S6K}$ have revealed, that in the absence of the amino-terminus, rapamycin can no longer block mitogen-induced T389 phosphorylation or kinase activation, demonstrating that inhibitory effects of rapamycin are not exerted through blocking the activation of an upstream kinase. However the mechanism by which rapamycin affects p70$^{S6K}$ activation through the amino-terminus remains unresolved. The failure to identify the immediate upstream p70$^{S6K}$ kinases as well as the mechanism by which rapamycin regulates p70$^{S6K}$ activation is largely explained by the multiple events required to bring about this response and the failure to reconstitute these events in vitro. This failure prompted the application of a number of complementary indirect approaches, including the use of inhibitors (Chung, J., *et al.* (1994) Nature 370, 71-75), dominant negative signalling molecules (Ming, X.F., *et al.* (1994) Nature 371, 426-429), and growth factor receptor mutants in the search for upstream components that regulate this response. However, as yet, this strategy has not yielded any additional insight into the identity of the upstream p70$^{S6K}$ kinases nor the mechanism for the selective effects of rapamycin on the kinase.

A powerful system which could complement the phosphorylation site studies described above, as well as resolve conflicting issues concerning the involvement of specific signalling molecules, is the use of developmental genetics. The use of genetic systems has proven extremely important in establishing the components that make up specific signal transduction pathways. For example, the application of a genetic approach to the study of the Drosophila sevenless mutant played a direct role in elucidating the signalling components of the MAP kinase pathway. In the case of p70$^{S6K}$, this system offers the additional attraction that in Drosophila, unlike yeast and slime moulds, the majority of ribosomal proteins contain a 5'TOP and their expression is selectively regulated at the translational level, in a manner similar to that found in mammals. Thus, the identification of a Drosophila p70$^{S6K}$ homologue could prove very important in identifying immediate upstream kinases and downstream targets as well as the mechanism by which rapamycin influences these responses.

## Summary of the Invention

The invention accordingly provid s *Drosophila melanogaster* p70$^{S6K}$, as well as nucleic acids encoding this kinase. The sequence of *Drosophila* p70$^{S6K}$ and the gene

encoding it are represented in SEQ ID No. 2 and 1 respectively. The invention moreover provides mutated forms of *Drosophila* p70$^{S6K}$, including constitutively active and dominant negative forms thereof, which are useful in the study of p70$^{S6K}$ activity. Furthermore, the invention provides expression systems which produce *Drosophila* p70$^{S6K}$ in *Drosophila* and other organisms, and in particular systems in which expression of *Drosophila* p70$^{S6K}$ has been modulated so as to facilitate the study of its activity.

Detailed Description of the Invention

Polypeptides according to the invention include *Drosophila* p70$^{S6K}$ and derivatives thereof which retain at least one common structural determinant of *Drosophila* p70$^{S6K}$.

"Common structural determinant" means that the derivative in question possesses at least one structural feature of *Drosophila* p70$^{S6K}$. Structural features includes possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against a naturally occurring or denatured *Drosophila* p70$^{S6K}$ polypeptide or fragment thereof, possession of amino acid sequence identity with *Drosophila* p70$^{S6K}$ and features having a common structure/function relationship. Thus *Drosophila* p70$^{S6K}$ as provided by the present invention includes splice variants encoded by mRNA generated by alternative splicing of a primary transcript, amino acid mutants, glycosylation variants and other covalent derivatives of *Drosophila* p70$^{S6K}$ which retain the physiological and/or physical properties of *Drosophila* p70$^{S6K}$. Exemplary derivatives include molecules wherein the protein of the invention is covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid. Such a moiety may be a detectable moiety such as an enzyme or a radioisotope. Further included are naturally occurring variants of *Drosophila* p70$^{S6K}$ found within a particular species. Such a variant may be encoded by a related gene of the same gene family, by an allelic variant of a particular gene, or represent an alternative splicing variant of the *Drosophila* p70$^{S6K}$ gene.

Derivatives which retain common structural features can be fragments of *Drosophila* p70$^{S6K}$. Fragments of *Drosophila* p70$^{S6K}$ comprise individual domains thereof, as well as smaller polypeptides derived from the domains. Preferably, smaller polypeptides derived from *Drosophila* p70$^{S6K}$ according to the invention define a single feature which is characteristic of *Drosophila* p70$^{S6K}$. Fragments may in theory be almost any size, as long as they retain one feature of *Drosophila* p70$^{S6K}$. Preferably, fragments will be between 5 and

350 amino acids in length. Longer fragments are regarded as truncations of the full-length *Drosophila* p70$^{S6K}$ and generally encompassed by the term "*Drosophila* p70$^{S6K}$ ".

Derivatives of *Drosophila* p70$^{S6K}$ also comprise mutants thereof, which may contain amino acid deletions, additions or substitutions, subject to the requirement to maintain at least one feature characteristic of *Drosophila* p70$^{S6K}$. Thus, conservative amino acid substitutions may be made substantially without altering the nature of *Drosophila* p70$^{S6K}$, as may truncations from the 5' or 3' ends. Deletions and substitutions may moreover be made to the fragments of *Drosophila* p70$^{S6K}$ comprised in the invention. *Drosophila* p70$^{S6K}$ mutants may be produced from a DNA encoding *Drosophila* p70$^{S6K}$ which has been subjected to *in vitro* mutagenesis, for example resulting in an addition, exchange and/or deletion of one or more amino acids. For example, substitutional, deletional or insertional variants of *Drosophila* p70$^{S6K}$ can be prepared by recombinant methods and screened for immuno-crossreactivity with the native forms of *Drosophila* p70$^{S6K}$.

The invention particularly comprises mutants of *Drosophila* p70$^{S6K}$ in which activity has been enhanced or reduced by amino acid substitution. Generally, the preferred substitutions are made at the residues homologous to those which have previously been indicated to be important in mouse p70$^{S6K}$. In the case of a "homologous" amino acid residue, it is intended to indicate that the homologous amino acids are present in a similar positional context and perform a similar role in different mouse and *Drosophila* enzymes. Thus, $T_{389}$, $T_{229}$ and $S_{371}$ in mouse p70$^{S6K}$ are homologous to $T_{389}$, $T_{238}$ and $S_{380}$ in *Drosophila* p70$^{S6K}$. $T_{389}$ is particularly indicated for mutation to an acidic amino acid residue in order to produce a constitutively active kinase.

Preferably, the $T_{389}$ mutation is combined with one or more additional serine or threonine substitutions involving insertion of an acidic amino acid in place thereof. For example, combination of the $T_{389}$->E with one or more of the $D_3E$ mutations is particularly advantageous.

The $D_3E$ mutations as herein defined comprises the mutations previously described in mammalian p70$^{S6K}$ by Ferrari *et al.*, (1993) J. Biol. Chem. **268**, 16091-16094, and equivalent mutations in *Drosophila* kinase. These mutations consist in the conversion of homologues of $S_{411}$, $S_{418}$ and $S_{424}$ to D and the conversion of the homologue of$T_{421}$ to E. The combination of any one or more of these mutations with the $T_{389}$->E mutation further increases the basal activity of p70$^{S6K}$ and imparts rapamycin resistance thereto. Preferably, the $T_{389}$->E mutation is combined with all of the $D_3E$ mutations.

Moreover, mutations may be effected at sites homologous to $S_{404}$ on $p70^{S6K}$. In general, threonine residues are converted to glutamic acid and serine residues are converted to aspartic acid.

Interestingly, although $T_{389}$ is the major rapamycin-sensitive site involved in the regulation of $p70^{S6K}$, it is not the only site involved in $p70^{S6K}$ activation. A second site which must be phosphorylated for $p70^{S6K}$ activation is $T_{229}$ ($T_{236}$). Dephosphorylation of this site results in immediate loss of any kinase activity. Moreover, mutation of this site to an acidic amino acid results in irreversible loss of kinase activity. Phosphorylation of $T_{229}$ is itself dependent on phosphorylation of $T_{389}$. Thus, a regulatory $p70^{S6K}$ kinase acts on $T_{389}$, phosphorylation of this site being responsible for activation of a further $p70^{S6K}$ kinase which phosphorylates $T_{229}$.

The invention accordingly provides *Drosophila* $p70^{S6K}$ comprising the $D_3E$ and $T_{389}$->E homologous mutations. Such a kinase enzyme is useful as a tool for identifying $p70^{S6K}$ kinases responsible for both regulation of the $p70^{S6K}$ related kinase and direct activation thereof through $T_{229}$.

Moreover, the invention provides Drosophila $p70^{S6K}$ comprising a T229->A and /or a K100->E homologous mutation. These mutants are dominant negative mutants of $p70^{S6K}$, irreversibly inactive and capable of preventing activation of endogenous $p70^{S6K}$ in a cell by competing therewith for factors essential for $p70^{S6K}$ activation. The K100 residue lies in the ATP binding site of $p70^{S6K}$. The dominant negative mutants compete for the upstream kinase kinase enzyme responsible for $p70^{S6K}$ activation and prevent its becoming available to phosphorylate endogenous $p70^{S6K}$ and other downstream targets. The mutants are useful as tools for defining the $p70^{S6K}$ signalling pathway, and as agents for blocking the upstream kinase kinase.

The fragments, mutants and other derivatives of *Drosophila* $p70^{S6K}$ preferably retain substantial homology with *Drosophila* $p70^{S6K}$. As used herein, "homology" means that the two entities share sufficient characteristics for the skilled person to determine that they are similar in origin and function. Preferably, homology is used to refer to sequence identity. Thus, the derivatives of *Drosophila* $p70^{S6K}$ preferably retain substantial sequence identity with the sequence of SEQ ID No. 2.

"Substantial homology", where homology indicates sequence identity, means more than 65% sequence identity, preferably more than 75% sequence identity and most preferably a sequence identity of 90% or more.

In still another aspect of the invention, the nucleic acid is DNA and further comprises a replicable vector comprising the nucleic acid encoding *Drosophila* p70$^{S6K}$ operably linked to control sequences recognised by a host transformed by the vector. Furthermore the invention provides host cells transformed with such a vector and a method of using a nucleic acid encoding *Drosophila* p70$^{S6K}$ to effect the production of *Drosophila* p70$^{S6K}$, comprising expressing *Drosophila* p70$^{S6K}$ nucleic acid in a culture of the transformed host cells and, if desired, recovering *Drosophila* p70$^{S6K}$ from the host cell culture.

Additionally, the present invention relates to isolated *Drosophila* p70$^{S6K}$ proteins and derivatives thereof encoded by the above-described nucleic acids.

In accordance with the present invention, there are provided isolated nucleic acids encoding *Drosophila* p70$^{S6K}$, or fragments thereof. In particular, the invention provides a DNA molecule encoding *Drosophila* p70$^{S6K}$, or a fragment thereof. By definition, such a DNA comprises a coding single stranded DNA, a double stranded DNA of said coding DNA and complementary DNA thereto, or this complementary (single stranded) DNA itself. An exemplary nucleic acid encoding *Drosophila* p70$^{S6K}$ is represented in SEQ ID No. 1.

Isolated *Drosophila* p70$^{S6K}$ nucleic acid includes nucleic acid that is free from at least one contaminant nucleic acid with which it is ordinarily associated in the natural source of *Drosophila* p70$^{S6K}$ nucleic acid or in crude nucleic acid preparations, such as DNA libraries and the like. Isolated nucleic acid thus is present in other than in the form or setting in which it is found in nature. However, isolated *Drosophila* p70$^{S6K}$ encoding nucleic acid includes *Drosophila* p70$^{S6K}$ nucleic acid in ordinarily *Drosophila* p70$^{S6K}$-expressing cells where the nucleic acid is in a chromosomal location different from that of natural cells or is otherwise flanked by a different DNA sequence than that found in nature.

The preferred sequence encoding *Drosophila* p70$^{S6K}$ is that having substantially the same nucleotide sequence as the coding sequences in SEQ ID No. 1, with the nucleic acid having the same sequence as the coding sequence in SEQ ID No. 1 being most preferred. As used herein, nucleotide sequences which are substantially the same share at least about 90 % identity. However, in the case of splice variants having e.g. an additional exon sequence homology may be lower.

The nucleic acids of the invention, whether used as probes or otherwise, are preferably substantially homologous to the sequence of *Drosophila* p70$^{S6K}$ as shown in SEQ ID No. 1. The terms "substantially" and "homologous" are used as hereinbefore defin d with reference to the *Drosophila* p70$^{S6K}$ polypeptide.

Preferably, nucleic acids according to the invention are fragments of the *Drosophila* $p70^{S6K}$-encoding s quence, or derivatives thereof as hereinbefore defined in relation to polypeptides. Fragments of the nucleic acid sequence of a few nucleotides in length, preferably 5 to 150 nucleotides in length, are especially useful as probes.

Exemplary nucleic acids can alternatively be characterised as those nucleotide sequences which encode a *Drosophila* $p70^{S6K}$ protein and hybridise to the DNA sequences set forth SEQ ID No. 1, or a selected fragment of said DNA sequence. Preferred are such sequences encoding *Drosophila* $p70^{S6K}$ which hybridise under high-stringency conditions to the sequence of SEQ ID No. 1.

Stringency of hybridisation refers to conditions under which polynucleic acids hybrids are stable. Such conditions are evident to those of ordinary skill in the field. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature ($T_m$) of the hybrid which decreases approximately 1 to 1.5°C with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridisation reaction is performed under conditions of higher stringency, followed by washes of varying stringency.

As used herein, high stringency refers to conditions that permit hybridisation of only those nucleic acid sequences that form stable hybrids in 1 M $Na^+$ at 65-68 °C. High stringency conditions can be provided, for example, by hybridisation in an aqueous solution containing 6x SSC, 5x Denhardt's, 1 % SDS (sodium dodecyl sulphate), 0.1 M sodium pyrophosphate and 0.1 mg/ml denatured salmon sperm DNA as non specific competitor. Following hybridisation, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridisation temperature in 0.2 - 0.1x SSC, 0.1 % SDS.

Moderate stringency refers to conditions equivalent to hybridisation in the above described solution but at about 60-62°C. In that case the final wash is performed at the hybridisation temperature in 1x SSC, 0.1 % SDS.

Low stringency refers to conditions equivalent to hybridisation in the above described solution at about 50-52°C. In that case, the final wash is performed at the hybridisation temperature in 2x SSC, 0.1 % SDS.

It is understood that these conditions may be adapted and duplicated using a variety of buffers, e.g. formamide-based buffers, and temperatures. Denhardt's solution and SSC are well known to those of skill in the art as are other suitable hybridisation buffers (see, e.g. Sambrook, *et al.*, eds. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York or Ausubel, *et al.*, eds. (1990) Current Protocols in

Molecular Biology, John Wiley & Sons, Inc.). Optimal hybridisation conditions have to be determined empirically, as the length and the GC content of the probe also play a role.

Given the guidance provided herein, the nucleic acids of the invention are obtainable according to methods well known in the art. For example, a DNA of the invention is obtainable by chemical synthesis, using polymerase chain reaction (PCR) or by screening a genomic library or a suitable cDNA library prepared from a source believed to possess *Drosophila* p70$^{S6K}$ and to express it at a detectable level.

Chemical methods for synthesis of a nucleic acid of interest are known in the art and include triester, phosphite, phosphoramidite and H-phosphonate methods as well as oligonucleotide synthesis on solid supports. These methods may be used if the entire nucleic acid sequence of the nucleic acid is known, or the sequence of the nucleic acid complementary to the coding strand is available. Alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue.

An alternative means to isolate the gene encoding *Drosophila* p70$^{S6K}$ is to use PCR technology, for example as described in section 14 of Sambrook et al., 1989. This method requires the use of oligonucleotide probes that will hybridise to *Drosophila* p70$^{S6K}$ nucleic acid. Strategies for selection of oligonucleotides are described below.

Libraries are screened with probes or analytical tools designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries suitable means include monoclonal or polyclonal antibodies that recognise and specifically bind to *Drosophila* p70$^{S6K}$ ; oligonucleotides of about 20 to 80 bases in length that encode *Drosophila* p70$^{S6K}$ cDNA; and/or complementary or homologous cDNAs or fragments thereof that encode the same or a hybridising gene. Appropriate probes for screening genomic DNA libraries include, but are not limited to oligonucleotides, cDNAs or fragments thereof that encode the same or hybridising DNA; and/or homologous genomic DNAs or fragments thereof.

A nucleic acid encoding *Drosophila* p70$^{S6K}$ may be isolated by screening suitable cDNA or genomic libraries under suitable hybridisation conditions with a probe, i.e. a nucleic acid disclosed herein including oligonucleotides derivable from the sequences set forth in SEQ ID NO. 1. Suitable libraries can be prepared from *Drosophila* by standard methodology.

As used herein, a probe is e.g. a single-stranded DNA or RNA that has a sequence of nucleotides that includes between 10 and 50, preferably between 15 and 30 and most

preferably at least about 20 contiguous bases that are the same as (or the complement of) an equivalent or greater number of contiguous bases set forth in SEQ ID No. 1. The nucleic acid sequences selected as probes should be of sufficient length and sufficiently unambiguous so that false positive results are minimised. The nucleotide sequences are usually based on conserved or highly homologous nucleotide sequences or regions of *Drosophila* $p70^{S6K}$. The nucleic acids used as probes may be degenerate at one or more positions. The use of degenerate oligonucleotides may be of particular importance where a library is screened from a species in which preferential codon usage in that species is not known.

Preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode ligand binding sites, such as the catalytically active site, and the like. For example, either the full-length cDNA clone disclosed herein or fragments thereof can be used as probes. Preferably, nucleic acid probes of the invention are labelled with suitable label means for ready detection upon hybridisation. For example, a suitable label means is a radiolabel. The preferred method of labelling a DNA fragment is by incorporating $\alpha^{32P}$ dATP with the Klenow fragment of DNA polymerase in a random priming reaction, as is well known in the art. Oligonucleotides are usually end-labelled with $\gamma^{32P}$-labelled ATP and polynucleotide kinase. However, other methods (e.g. non-radioactive) may also be used to label the fragment or oligonucleotide, including e.g. enzyme labelling, fluorescent labelling with suitable fluorophores and biotinylation.

After screening the library, e.g. with a portion of DNA including substantially the entire *Drosophila* $p70^{S6K}$-encoding sequence or a suitable oligonucleotide based on a portion of said DNA, positive clones are identified by detecting a hybridisation signal; the identified clones are characterised by restriction enzyme mapping and/or DNA sequence analysis, and then examined, e.g. by comparison with the sequences set forth herein, to ascertain whether they include DNA encoding a complete *Drosophila* $p70^{S6K}$ (i.e., if they include translation initiation and termination codons). If the selected clones are incomplete, they may be used to rescreen the same or a different library to obtain overlapping clones. If the library is genomic, then the overlapping clones may include exons and introns. If the library is a cDNA library, then the overlapping clones will include an open reading frame. In both instances, complete clones may be identified by comparison with the DNAs and deduced amino acid sequences provided h rein.

In order to detect any abnormality of endogenous *Drosophila* p70$^{S6K}$, genetic screening may be carried out using the nucleotide sequences of the invention as hybridisation probes. Also, based on the nucleic acid sequences provided herein antisense-type therapeutic agents may be designed.

It is envisaged that the nucleic acid of the invention can be readily modified by nucleotide substitution, nucleotide deletion, nucleotide insertion or inversion of a nucleotide stretch, and any combination thereof. Such mutants can be used e.g. to produce a *Drosophila* p70$^{S6K}$ mutant that has an amino acid sequence differing from the *Drosophila* p70$^{S6K}$ sequences as found in nature. Mutagenesis may be predetermined (site-specific) or random. A mutation which is not a silent mutation must not place sequences out of reading frames and preferably will not create complementary regions that could hybridise to produce secondary mRNA structure such as loops or hairpins.

If required, nucleic acids encoding *Drosophila* p70$^{S6K}$-related enzymes may be cloned from *Drosophila* spp. according to established procedures using probes derived from *Drosophila* p70$^{S6K}$ itself or any of the published sequences of related D-ala-D-ala ligases. In particular, such DNAs can be prepared by:

a) isolating mRNA from suitable *Drosophila* cells, selecting the desired mRNA, for example by hybridisation with a DNA probe or by expression in a suitable expression system and screening for expression of the desired polypeptide, preparing single-stranded cDNA complementary to that mRNA, then double-stranded cDNA therefrom, or

b) isolating cDNA from a cDNA library and selecting the desired cDNA, for example using a DNA probe or using a suitable expression system and screening for expression of the desired polypeptide,

c) incorporating the double-stranded DNA of step a) or b) into an appropriate expression vector,

d) transforming appropriate host cells with the vector and isolating the desired DNA.

Messenger RNA (step a) is isolated by known methods. Isolation methods involve, for example, homogenizing cells in the presence of a detergent and a ribonuclease inhibitor, for example heparin, guanidinium isothiocyanate or mercaptoethanol, extracting the mRNA with a chloroform-phenol mixture, optionally in the presence of salt and buffer solutions, detergents and/or cation chelating agents, and precipitating mRNA from the remaining aqueous, salt-containing phase with ethanol, isopropanol or the like. The isolated mRNA may be further purified by centrifuging in a caesium chloride gradient followed by ethanol precipitation and/or by chromatographic methods, for xample affinity chromatography, for

example chromatography on oligo(dT) cellulose or on oligo(U) sepharose if it is polyadenylated. Preferably, such purified total mRNA is fractionated according to size by gradient centrifugation, for example in a linear sucrose gradient, or chromatography on suitable size fractionation columns, for example on agarose gels.

The desired mRNA is selected by screening the mRNA directly with a DNA probe, or by translation in suitable cells or cell-free systems and screening the obtained polypeptides.

The selection of the desired mRNA is preferably achieved using a DNA hybridisation probe, thereby avoiding the additional step of translation. Suitable DNA probes are DNAs of known nucleotide sequence consisting of at least 17 nucleotides derived from DNAs encoding *Drosophila* $p70^{S6K}$ or a related ligase.

Synthetic DNA probes are synthesised according to known methods as detailed hereinbelow, preferably by stepwise condensation using the solid phase phosphotriester, phosphite triester or phosphoramidite method, for example the condensation of dinucleotide coupling units by the phosphotriester method. These methods are adapted to the synthesis of mixtures of the desired oligonucleotides by using mixtures of two, three or four nucleotides dA, dC, dG and/or dT in protected form or the corresponding dinucleotide coupling units in the appropriate condensation step as described by Y. Ike et al. (Nucleic Acids Research 11, 477, 1983).

For hybridisation, the DNA probes are labelled, for example radioactively labelled by the well known kinase reaction. The hybridisation of the size-fractionated mRNA with the DNA probes containing a label is performed according to known procedures, i.e. in buffer and salt solutions containing adjuncts, for example calcium chelators, viscosity regulating compounds, proteins, irrelevant DNA and the like, at temperatures favouring selective hybridisation, for example between 0°C and 80°C, for example between 25°C and 50°C or around 65°C, preferably at around 20° lower than the hybrid double-stranded DNA melting temperature.

Fractionated mRNA may be translated in cells, for example frog oocytes, or in cell-free systems, for example in reticulocyte lysates or wheat germ extracts. The obtained polypeptides are screened for D-ala-D-ala ligase activity or for reaction with antibodies raised against the *Drosophila* $p70^{S6K}$ related ligase, for example in an immunoassay, for example radioimmunoassay, enzyme immunoassay or immunoassay with fluorescent markers. Such immunoassays and the preparation of polyclonal and monoclonal antibodies are well known in the art and are applied accordingly.

The preparation of a single-stranded complementary DNA (cDNA) from the selected mRNA template is well known in the art, as is the preparation of a double-stranded DNA from a single-stranded DNA. The mRNA template is incubated with a mixture of deoxynucleoside triphosphates, optionally radioactively labelled deoxynucleoside triphosphates (in order to be able to screen the result of the reaction), a primer sequence such as an oligo-dT residue hybridising with the poly(A) tail of the mRNA and a suitable enzyme such as a reverse transcriptase for example from avian myeloblastosis virus (AMV). After degradation of the template mRNA for example by alkaline hydrolysis, the cDNA is incubated with a mixture of deoxynucleoside triphosphates and a suitable enzyme to give a double-stranded DNA. Suitable enzymes are for instance a reverse transcriptase, the Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase. Usually, a hairpin loop structure formed spontaneously by the single-stranded cDNA acts as a primer for the synthesis of the second strand. This hairpin structure is removed by digestion with S1 nuclease. Alternatively, the 3'-end of the single-stranded DNA is first extended by homopolymeric deoxynucleotide tails prior to the hydrolysis of the mRNA template and the subsequent synthesis of the second cDNA strand.

In the alternative, double-stranded cDNA is isolated from a cDNA library and screened for the desired cDNA (step b). The cDNA library is constructed by isolating mRNA from *Drosophila* cells, and preparing single-stranded and double-stranded cDNA therefrom as described above. This cDNA is digested with suitable restriction endonucleases and incorporated into λ phage, for example λ charon 4A or λ gt11 following established procedures. The cDNA library replicated on nitrocellulose membranes is screened by using a DNA probe as described hereinbefore, or expressed in a suitable expression system and the obtained polypeptides screened for reaction with an antibody specific for *Drosophila* $p70^{S6K}$.

A variety of methods are known in the art for the incorporation of double-stranded cDNA into an appropriate vector (step c). For example, complementary homopolymer tracts may be added to the double-stranded DNA and the vector DNA by incubation in the presence of the corresponding deoxynucleoside triphosphates and an enzyme such as terminal deoxynucleotidyl transferase. The vector and double-stranded DNA are then joined by base pairing between the complementary homopolymeric tails and finally ligated by specific joining enzymes such as ligases. Other possibilities are the addition of synthetic

linkers to the termini of the double-stranded DNA, or the incorporation of the double-stranded DNA into the vector by blunt- or staggered-end ligation.

The transformation of appropriate host cells with the obtained hybrid vector (step d) and the selection of transformed host cells (step e) are well known in the art. Hybrid vectors and host cells may be particularly suitable for the production of DNA, or for the production of the desired *Drosophila* $p70^{S6K}$.

The isolation of the desired DNA is achieved by methods known in the art, for example extraction with phenol and/or chloroform or on glass beads. Optionally, the DNA can be further manipulated for example by treatment with mutagenic agents to obtain mutants, or by digestion with restriction enzymes to obtain fragments, modify one or both termini to facilitate incorporation into the vector.

The cDNA or genomic DNA encoding native or mutant *Drosophila* $p70^{S6K}$ can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well within the skill of the artisan. Many vectors are available, and selection of appropriate vector will depend on the intended use of the vector, i.e. whether it is to be used for DNA amplification or for DNA expression, the size of the DNA to be inserted into the vector, and the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: an origin of replication, one or more marker genes, an enhancer element, a promoter, a transcription termination sequence and a signal sequence.

Both expression and cloning vectors generally contain nucleic acid sequence that enable the vector to replicate in one or more selected host cells. Typically in cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (e.g. SV 40, polyoma, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors unless

thes are used in mammalian cells competent for high level DNA replication, such as COS cells.

Most expression vectors are shuttle vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells even though it is not capable of replicating independently of the host cell chromosome. DNA may also be replicated by insertion into the host genome. DNA can be amplified by PCR and be directly transfected into the host cells without any replication component.

Advantageously, an expression and cloning vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available from complex media.

As to a selective gene marker appropriate for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker gene. Suitable markers for yeast are, for example, those conferring resistance to antibiotics G418, hygromycin or bleomycin, or provide for prototrophy in an auxotrophic yeast mutant, for example the URA3, LEU2, LYS2, TRP1, or HIS3 gene.

Since the replication of vectors is conveniently done in *E. coli*, an *E. coli* genetic marker and an *E. coli* origin of replication are advantageously included. These can be obtained from *E. coli* plasmids, such as pBR322, Bluescript® vector or a pUC plasmid, e.g. pUC18 or pUC19, which contain both *E. coli* replication origin and *E. coli* genetic marker conferring resistance to antibiotics, such as ampicillin.

Suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up *Drosophila* $p70^{S6K}$ nucleic acid, such as dihydrofolate reductase (DHFR, methotrexate resistance), thymidine kinase, or genes conferring resistance to G418 or hygromycin. The mammalian cell transformants are placed under selection pressure which only those transformants which have taken up and are expressing the marker are uniquely adapted to survive. In the case of a DHFR or glutamine synthase (GS) marker, selection pressure can be imposed by culturing the transformants under conditions in which the pressure is progressively increased, thereby leading to

amplification (at its chromosomal integration site) of both the selection gene and the linked DNA that encodes *Drosophila* p70$^{S6K}$. Amplification is the process by which genes in greater demand for the production of a protein critical for growth, together with closely associated genes which may encode a desired protein, are reiterated in tandem within the chromosomes of recombinant cells. Increased quantities of desired protein are usually synthesised from thus amplified DNA.

An expression vector includes any vector capable of expressing *Drosophila* p70$^{S6K}$ nucleic acids that are operatively linked with regulatory sequences, such as promoter regions, that are capable of expression of such DNAs. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector, that upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those with ordinary skill in the art and include those that are replicable in eukaryotic and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. For example, DNAs encoding *Drosophila* p70$^{S6K}$ may be inserted into a vector suitable for expression of cDNAs in mammalian cells, e.g. a CMV enhancer-based vector such as pEVRF (Matthias, *et al.*, (1989) NAR 17, 6418).

Particularly useful for practising the present invention are expression vectors that provide for the transient expression of DNA encoding *Drosophila* p70$^{S6K}$ in mammalian cells. Transient expression usually involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector, and, in turn, synthesises high levels of *Drosophila* p70$^{S6K}$. For the purposes of the present invention, transient expression systems are useful e.g. for identifying *Drosophila* p70$^{S6K}$ mutants, to identify potential phosphorylation sites, or to characterise functional domains of the protein.

Construction of vectors according to the invention employs conventional ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the constructed plasmids is performed in a known fashion. Suitable methods for constructing expression vectors, preparing in vitro transcripts, introducing DNA into host cells, and performing analyses for assessing *Drosophila* p70$^{S6K}$ expression and function are known to those skill d in the art. Gene presence, amplification and/or expression may be measured in a sample dir ctly, for example, by conventional Southern blotting, Northern

blotting to quantitate the transcription of mRNA, dot blotting (DNA or RNA analysis), or in situ hybridisation, using an appropriately labelled probe based on a sequence provided herein. Those skilled in the art will readily envisage how these methods may be modified, if desired.

Expression and cloning vectors usually contain a promoter that is recognised by the host organism and is operably linked to *Drosophila* $p70^{S6K}$ nucleic acid. Such a promoter may be inducible or constitutive. The promoters are operably linked to DNA encoding *Drosophila* $p70^{S6K}$ by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native *Drosophila* $p70^{S6K}$ promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of *Drosophila* $p70^{S6K}$ DNA.

Promoters suitable for use with prokaryotic hosts include, for example, the β-lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (trp) promoter system and hybrid promoters such as the tac promoter. Their nucleotide sequences have been published, thereby enabling the skilled worker operably to ligate them to DNA encoding *Drosophila* $p70^{S6K}$, using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems will also generally contain a Shine-Delgarno sequence operably linked to the DNA encoding *Drosophila* $p70^{S6K}$.

Moreover, the *Drosophila* $p70^{S6K}$ gene according to the invention preferably includes a secretion sequence in order to facilitate secretion of the polypeptide from bacterial hosts, such that it will be produced as a soluble native peptide rather than in an inclusion body. The peptide may be recovered from the bacterial periplasmic space, or the culture medium, as appropriate.

Suitable promoting sequences for use with yeast hosts may be regulated or constitutive and are preferably derived from a highly expressed yeast gene, especially a *Saccharomyces cerevisiae* gene. Thus, the promoter of the TRP1 gene, the ADHI or ADHII gene, the acid phosphatase (PH05) gene, a promoter of the yeast mating pheromone genes coding for the α- or a-factor or a promoter derived from a gene encoding a glycolytic enzyme such as the promoter of the enolase, glyceraldehyde-3-phosphate dehydrogenase (GAP), 3-phospho glycerate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglyc rate mutase, pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase or glucokinase genes, or a promoter from the TATA binding protein (TBP) gene can be us d. Furthermore,

it is possible to use hybrid promoters comprising upstream activation sequences (UAS) of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene, for example a hybrid promoter including the UAS(s) of the yeast PH05 gene and downstream promoter elements including a functional TATA box of the yeast GAP gene (PH05-GAP hybrid promoter). A suitable constitutive PHO5 promoter is e.g. a shortened acid phosphatase PH05 promoter devoid of the upstream regulatory elements (UAS) such as the PH05 (-173) promoter element starting at nucleotide -173 and ending at nucleotide -9 of the PH05 gene.

*Drosophila* p70$^{S6K}$ gene transcription from vectors in mammalian hosts may be controlled by promoters derived from the genomes of viruses such as polyoma virus, adenovirus, fowlpox virus, bovine papilloma virus, avian sarcoma virus, cytomegalovirus (CMV), a retrovirus and Simian Virus 40 (SV40), from heterologous mammalian promoters such as the actin promoter or a very strong promoter, e.g. a ribosomal protein promoter, and from the promoter normally associated with *Drosophila* p70$^{S6K}$ sequence, provided such promoters are compatible with the host cell systems.

Transcription of a DNA encoding *Drosophila* p70$^{S6K}$ by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are relatively orientation and position independent. Many enhancer sequences are known from mammalian genes (e.g. elastase and globin). However, typically one will employ an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270) and the CMV early promoter enhancer. The enhancer may be spliced into the vector at a position 5' or 3' to *Drosophila* p70$^{S6K}$ DNA, but is preferably located at a site 5' from the promoter.

Suitable eukaryotic host cells for expression of *Drosophila* p70$^{S6K}$ include yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms will also contain sequences necessary for the termination of transcription and for stabilising the mRNA. Such sequences are commonly available from the 5' and 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding *Drosophila* p70$^{S6K}$.

In addition to being useful for the production of recombinant *Drosophila* p70$^{S6K}$ protein, these nucleic acids are also useful as probes, thus readily enabling those skilled in the art to identify and/or isolate nucleic acid encoding *Drosophila* p70$^{S6K}$ or related enzymes

from other *Drosophila* spp. The nucleic acid may be unlabelled or labelled with a detectable moiety. Furthermore, nucleic acid according to the invention is useful e.g. in a method determining the presence of *Drosophila* $p70^{S6K}$-specific nucleic acid, said method comprising hybridising the DNA (or RNA) encoding (or complementary to) *Drosophila* $p70^{S6K}$ to test sample nucleic acid and determining the presence of *Drosophila* $p70^{S6K}$. In another aspect, the invention provides nucleic acid sequence that is complementary to, or hybridises under stringent conditions to, a nucleic acid sequence encoding *Drosophila* $p70^{S6K}$.

The invention also provides a method for amplifying a nucleic acid test sample comprising priming a nucleic acid polymerase (chain) reaction with nucleic acid (DNA or RNA) encoding (or complementary to) *Drosophila* $p70^{S6K}$.

In accordance with another embodiment of the present invention, there are provided cells containing the above-described nucleic acids. Such host cells such as prokaryote, yeast and higher eukaryote cells may be used for replicating DNA and producing *Drosophila* $p70^{S6K}$. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, such as *E. coli*, e.g. *E. coli* K-12 strains, DH5α and HB101, or Bacilli, such as *B. subtilis*. Further hosts suitable for *Drosophila* $p70^{S6K}$ encoding vectors include eukaryotic microbes such as filamentous fungi or yeast, e.g. Saccharomyces cerevisiae. Higher eukaryotic cells include insect and vertebrate cells, particularly mammalian cells. In recent years propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are epithelial or fibroblastic cell lines such as Chinese hamster ovary (CHO) cells, NIH 3T3 cells, HeLa cells or 293T cells. The host cells referred to in this disclosure comprise cells in *in vitro* culture as well as cells that are within a host animal.

DNA may be stably incorporated into cells or may be transiently expressed using methods known in the art. Stably transfected mammalian cells may be prepared by transfecting cells with an expression vector having a selectable marker gene, and growing the transfected cells under conditions selective for cells expressing the marker gene. To prepare transient transfectants, mammalian cells are transfected with a reporter gene to monitor transfection efficiency.

To produce such stably or transiently transfected cells, the cells should be transfected with a sufficient amount of *Drosophila* $p70^{S6K}$-encoding nucleic acid to form *Drosophila*

p70$^{S6K}$. The precise amounts of DNA encoding *Drosophila* p70$^{S6K}$ may be empirically determined and optimised for a particular cell and assay.

Host cells are transfected or, preferably, transformed with the above-captioned expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Heterologous DNA may be introduced into host cells by any method known in the art, such as transfection with a vector encoding a heterologous DNA by the calcium phosphate coprecipitation technique or by electroporation. Numerous methods of transfection are known to the skilled worker in the field. Successful transfection is generally recognised when any indication of the operation of this vector occurs in the host cell. Transformation is achieved using standard techniques appropriate to the particular host cells used.

Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are well known in the art (see, e.g. Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press).

Transfected or transformed cells are cultured using media and culturing methods known in the art, preferably under conditions, whereby *Drosophila* p70$^{S6K}$ encoded by the DNA is expressed. The composition of suitable media is known to those in the art, so that they can be readily prepared. Suitable culturing media are also commercially available.

The polypeptide according to the invention may advantageously be expressed in insect cell systems. Insect cells suitable for use in the method of the invention include, in principle, any lepidopteran cell which is capable of being transformed with an expression vector and expressing heterologous proteins encoded thereby. In particular, use of the Sf cell lines, such as the *Spodoptera frugiperda* cell line IPBL-SF-21 AE (Vaughn *et al.*, (1977) In Vitro, **13**, 213-217) is preferred. The derivative cell line Sf9 is particularly preferred. However, other cell lines, such as *Tricoplusia ni* 368 (Kurstack and Marmorosch, (1976) Invertebrate Tissue Culture Applications in Medicine, Biology and Agriculture. Academic Press, New York, USA) may be employed. These cell lines, as well as other insect cell lines suitable for use in the invention, are commercially available (e.g. from Stratagene, La Jolla, CA, USA).

As well as expression in insect cells in culture, the invention also comprises the expression of heterologous proteins in whole insect organisms. The use of virus vectors

such as baculovirus allows infection of entire insects, which are in some ways easier to grow than cultured cells as they have fewer requirements for special growth conditions. Large insects, such as silk moths, provide a high yield of heterologous protein. The protein can be extracted from the insects according to conventional extraction techniques.

Expression vectors suitable for use in the invention include all vectors which are capable of expressing foreign proteins in insect cell lines. In general, vectors which are useful in mammalian and other eukaryotic cells are also applicable to insect cell culture. Baculovirus vectors, specifically intended for insect cell culture, are especially preferred and are widely obtainable commercially (e.g. from Invitrogen and Clontech). Other virus vectors capable of infecting insect cells are known, such as Sindbis virus (Hahn *et al.*, (1992) PNAS (USA) **89**, 2679-2683). The baculovirus vector of choice (reviewed by Miller (1988) Ann. Rev. Microbiol. **42**, 177-199) is *Autographa californica* multiple nuclear polyhedrosis virus, AcMNPV.

Typically, the heterologous gene replaces at least in part the polyhedrin gene of AcMNPV, since polyhedrin is not required for virus production. In order to insert the heterologous gene, a transfer vector is advantageously used. Transfer vectors are prepared in *E. coli* hosts and the DNA insert is then transferred to AcMNPV by a process of homologous recombination.

Expression systems encoding *Drosophila* $p70^{S6K}$ are useful for the study of $p70^{S6K}$ activity, particularly in the context of transgenic *Drosophila* flies. Preferred is a system in which $p70^{S6K}$ expression has been attenuated, particularly where this is achieved by means of transposon insertion. It has been determined, for instance, that mutant *fs(3)07084* (Karpen, G.H. & Spradling A.C. Genetics 132, 737-753 (1992); Spradling, A.C. *et al.* Proc. Natl. Acad. Sci. USA 92, 10824-10830 (1995)) contains a P-element insertion in the 5' region of the *Drosophila* $p70^{S6K}$ gene. Sequencing of the mutant shows that the insertion is localised 41 base pairs upstream of the *Drosophila* $p70^{S6K}$ AUG initiator codon.

Mutant files according to the invention have impaired $p70^{S6K}$ expression. Homozygotes for the *fs(3)07084* mutation show impaired and/or delayed oogenesis, due to low levels of $p70^{S6K}$ production as a result of attenuation of gene transcription by the transposon insertion. Mutations in which $p70^{S6K}$ expression is entirely eliminated are lethal in homozygous form.

Expression mutants of *Drosophila* $p70^{S6K}$, particularly those in which expression is severely attenuat d but not eliminated, are useful for the study of $p70^{S6K}$ activity. They

show increased sensitivity to modulated interaction of putative upstream signalling agents with the regulatory domains of p70$^{S6K}$, as well as modification of the downstream targets predicted to mediate its biological response. Thus, the invention also provides a method for assessing the ability of an agent to target *Drosophila* p70$^{S6K}$ activity comprising exposing a *Drosophila* p70$^{S6K}$ mutant as described herein to the agent, and judging the effect of the biological activity of p70$^{S6K}$.

In accordance with yet another embodiment of the present invention, there are provided antibodies specifically recognising and binding to *Drosophila* p70$^{S6K}$. For example, such antibodies may be generated against the *Drosophila* p70$^{S6K}$ having the amino acid sequences set forth in SEQ ID No. 2. Alternatively, *Drosophila* p70$^{S6K}$ or *Drosophila* p70$^{S6K}$ fragments (which may also be synthesised by *in vitro* methods) are fused (by recombinant expression or an *in vitro* peptidyl bond) to an immunogenic polypeptide and this fusion polypeptide, in turn, is used to raise antibodies against a *Drosophila* p70$^{S6K}$ epitope.

Anti-*Drosophila* p70$^{S6K}$ antibodies are recovered from the serum of immunised animals. Alternatively, monoclonal antibodies are prepared from cells *in vitro* or from *in vivo* immunised animals in conventional manner.

The antibodies of the invention are useful for studying *Drosophila* p70$^{S6K}$ localisation, screening of an expression library to identify nucleic acids encoding *Drosophila* p70$^{S6K}$ or the structure of functional domains, as well as for the purification of *Drosophila* p70$^{S6K}$, and the like.

Antibodies according to the invention may be whole antibodies of natural classes, such as IgE and IgM antibodies, but are preferably IgG antibodies. Moreover, the invention includes antibody fragments, such as Fab, F(ab')$_2$, Fv and ScFv. Small fragments, such Fv and ScFv, possess advantageous properties for diagnostic and therapeutic applications on account of their small size and consequent superior tissue distribution.

The antibodies according to the invention are especially indicated for diagnostic and therapeutic applications. Accordingly, they may be altered antibodies comprising an effector protein such as a toxin or a label. Especially preferred are labels which allow the imaging of the distribution of the antibody at the site of infection *in vivo*, or in a biopsy. Such labels may be radioactive labels or radioopaque labels, such as metal particles, which are readily visualisable within the body of a patient. Moreover, they may be fluorescent labels or other lab Is which are visualisable on tissue samples removed from patients.

R combinant DNA technology may be used to improve the antibodies of the invention. Thus, chimeric antibodies may be constructed in order to decrease the immunogenicity thereof in diagnostic or therapeutic applications. Moreover, immunogenicity may be minimised by humanising the antibodies by CDR grafting [see European Patent Application 0 239 400 (Winter)] and, optionally, framework modification [see international patent application WO 90/07861 (Protein Design Labs)].

Antibodies according to the invention may be obtained from animal serum, or, in the case of monoclonal antibodies or fragments thereof, produced in cell culture. Recombinant DNA technology may be used to produce the antibodies according to established procedure, in bacterial or preferable in mammalian cell culture. The selected cell culture system preferably secretes the antibody product.

Therefore, the present invention includes a process for the production of an antibody according to the invention comprising culturing a host, e.g. *E. coli* or a mammalian cell, which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding the antibody, and isolating said antibody.

Multiplication of hybridoma cells or mammalian host cells *in vitro* is carried out in suitable culture media, which are the customary standard culture media, for example Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium, optionally replenished by a mammalian serum, e.g. fetal calf serum, or trace elements and growth sustaining supplements, e.g. feeder cells such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages, 2-aminoethanol, insulin, transferrin, low density lipoprotein, oleic acid, or the like. Multiplication of host cells which are bacterial cells or yeast cells is likewise carried out in suitable culture media known in the art, for example for bacteria in medium LB, NZCYM, NZYM, NZM, Terrific Broth, SOB, SOC, 2 x YT, or M9 Minimal Medium, and for yeast in medium YPD, YEPD, Minimal Medium, or Complete Minimal Dropout Medium.

*In vitro* production provides relatively pure antibody preparations and allows scale-up to give large amounts of the desired antibodies. Techniques for bacterial cell, yeast or mammalian cell cultivation are known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilised or entrapped cell culture, e.g. in hollow fibres, microcapsules, on agarose microbeads or ceramic cartridges.

Large quantities of the desired antibodies can also be obtained by multiplying mammalian cells *in vivo*. For this purpose, hybridoma cells producing the desired antibodies are injected into histocompatible mammals to cause growth of antibody-producing tumours. Optionally, the animals are primed with a hydrocarbon, especially mineral oils such as pristane (tetramethyl-pentadecane), prior to the injection. After one to three weeks, the antibodies are isolated from the body fluids of those mammals. For example, hybridoma cells obtained by fusion of suitable myeloma cells with antibody-producing spleen cells from Balb/c mice, or transfected cells derived from hybridoma cell line Sp2/0 that produce the desired antibodies are injected intraperitoneally into Balb/c mice optionally pre-treated with pristane, and, after one to two weeks, ascitic fluid is taken from the animals.

The cell culture supernatants are screened for the desired antibodies, preferentially by immunofluorescent staining of cells expressing *Drosophila* $p70^{S6K}$, by immunoblotting, by an enzyme immunoassay, e.g. a sandwich assay or a dot-assay, or a radioimmunoassay.

For isolation of the antibodies, the immunoglobulins in the culture supernatants or in the ascitic fluid may be concentrated, e.g. by precipitation with ammonium sulphate, dialysis against hygroscopic material such as polyethylene glycol, filtration through selective membranes, or the like. If necessary and/or desired, the antibodies are purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose and/or (immuno-)affinity chromatography, e.g. affinity chromatography with *Drosophila* $p70^{S6K}$ protein covalently attached to a solid support or with Protein-A.

The invention further concerns hybridoma cells secreting the monoclonal antibodies of the invention. The preferred hybridoma cells of the invention are genetically stable, secrete monoclonal antibodies of the invention of the desired specificity and can be activated from deep-frozen cultures by thawing and recloning.

The invention also concerns a process for the preparation of a hybridoma cell line secreting monoclonal antibodies directed *Drosophila* $p70^{S6K}$, characterised in that a suitable mammal, for example a Balb/c mouse, is immunised with purified *Drosophila* $p70^{S6K}$ protein, an antigenic carrier containing purified *Drosophila* $p70^{S6K}$ or with cells bearing *Drosophila* $p70^{S6K}$, antibody-producing cells of the immunised mammal are fused with cells of a suitable myeloma cell line, the hybrid c lls obtained in the fusion are cloned, and cell clones secreting the desired antibodies are selected. For example spleen cells of Balb/c mice

immunised with cells bearing *Drosophila* p70$^{S6K}$ are fused with cells of the myeloma cell line PAI or the myeloma cell line Sp2/0-Ag14, the obtained hybrid cells are screened for secretion of the desired antibodies, and positive hybridoma cells are cloned.

Preferred is a process for the preparation of a hybridoma cell line, characterised in that Balb/c mice are immunised by injecting subcutaneously and/or intraperitoneally between $10^7$ and $10^8$ cells which express *Drosophila* p70$^{S6K}$ containing a suitable adjuvant several times, e.g. four to six times, over several months, e.g. between two and four months, and spleen cells from the immunised mice are taken two to four days after the last injection and fused with cells of the myeloma cell line PAI in the presence of a fusion promoter, preferably polyethylene glycol. Preferably the myeloma cells are fused with a three- to twentyfold excess of spleen cells from the immunised mice in a solution containing about 30 % to about 50 % polyethylene glycol of a molecular weight around 4000. After the fusion the cells are expanded in suitable culture media as described hereinbefore, supplemented with a selection medium, for example HAT medium, at regular intervals in order to prevent normal myeloma cells from overgrowing the desired hybridoma cells.

The invention also concerns recombinant DNAs comprising an insert coding for a heavy chain variable domain and/or for a light chain variable domain of antibodies directed to the extracellular domain of *Drosophila* p70$^{S6K}$ as described hereinbefore. By definition such DNAs comprise coding single stranded DNAs, double stranded DNAs consisting of said coding DNAs and of complementary DNAs thereto, or these complementary (single stranded) DNAs themselves.

Furthermore, DNA encoding a heavy chain variable domain and/or for a light chain variable domain of antibodies directed *Drosophila* p70$^{S6K}$ can be enzymatically or chemically synthesised DNA having the authentic DNA sequence coding for a heavy chain variable domain and/or for the light chain variable domain, or a mutant thereof. A mutant of the authentic DNA is a DNA encoding a heavy chain variable domain and/or a light chain variable domain of the above-mentioned antibodies in which one or more amino acids are deleted or exchanged with one or more other amino acids. Preferably said modification(s) are outside the CDRs of the heavy chain variable domain and/or of the light chain variable domain of the antibody. Such a mutant DNA is also intended to be a silent mutant wherein one or more nucleotides are replaced by other nucleotides with the new codons coding for the same amino acid(s). Such a mutant sequence is also a degenerate sequence. Degenerate sequences are degenerate within the meaning of the genetic code in that an unlimited number of nucleotides are replaced by other nucleotides without resulting in a

change of the amino acid sequence originally encoded. Such degen rate sequences may be useful due to their different restriction sites and/or frequency of particular codons which are preferred by the specific host, particularly *E. coli*, to obtain an optimal expression of the heavy chain murine variable domain and/or a light chain murine variable domain.

The term mutant is intended to include a DNA mutant obtained by in vitro mutagenesis of the authentic DNA according to methods known in the art.

For the assembly of complete tetrameric immunoglobulin molecules and the expression of chimeric antibodies, the recombinant DNA inserts coding for heavy and light chain variable domains are fused with the corresponding DNAs coding for heavy and light chain constant domains, then transferred into appropriate host cells, for example after incorporation into hybrid vectors.

The invention therefore also concerns recombinant DNAs comprising an insert coding for a heavy chain murine variable domain of an antibody directed *Drosophila* $p70^{S6K}$ fused to a human constant domain $\gamma$, for example $\gamma1$, $\gamma2$, $\gamma3$ or $\gamma4$, preferably $\gamma1$ or $\gamma4$. Likewise the invention concerns recombinant DNAs comprising an insert coding for a light chain murine variable domain of an antibody directed to *Drosophila* $p70^{S6K}$ fused to a human constant domain $\kappa$ or $\lambda$, preferably $\kappa$.

In another embodiment the invention pertains to recombinant DNAs coding for a recombinant DNA wherein the heavy chain variable domain and the light chain variable domain are linked by way of a DNA insert coding for a spacer group, optionally comprising a signal sequence facilitating the processing of the antibody in the host cell and/or a DNA coding for a peptide facilitating the purification of the antibody and/or a DNA coding for a cleavage site and/or a DNA coding for a peptide spacer and/or a DNA coding for an effector molecule.

The DNA coding for an effector molecule is intended to be a DNA coding for the effector molecules useful in diagnostic or therapeutic applications. Thus, effector molecules which are toxins or enzymes, especially enzymes capable of catalysing the activation of prodrugs, are particularly indicated. The DNA encoding such an effector molecule has the sequence of a naturally occurring enzyme or toxin encoding DNA, or a mutant thereof, and can be prepared by methods well known in the art.

The invention is described hereinbelow, for the purposes of illustration only, in the following examples.

MATERIALS AND METHODS

Nucleic Acid Isolation and Hybridisation.

Genomic DNA and total RNA are isolated from adult Canton S flies as previously described (Stewart, M.J. & Denell, R. (1993) Mol. Cell. Biol. 13, 2524-2535) DNA and RNA are fractionated on a 0.8% agarose gel in 0.5X TBE or a 0.7% agarose gel containing 2.2 M formaldehyde, 50mM MOPS, and 1mM EDTA, respectively, transferred to Hybond N membranes (Amersham) and hybridised as described. Final washes are carried out twice for 30 minutes at 68°C in 0.1X SSPE; 0.1% SDS. DNA probes are labelled using a random priming kit (Boehringer) to a specific activity of >109 cpm per ug.

cDNA Library Screening and Sequencing.

Genomic and cDNA libraries are screened employing hybridisation and washing conditions as above. A fragment of the rat $p70^{S6K}$ 2B4 clone encompassing nucleotides 42 to 431 (Reinhard, C., *et al.* (1992) Proc. Natl. Acad. Sci. USA 89, 4052-4056) is used to screen a Drosophila genomic library constructed from Dr(2R)SB1/CyO flies (Baumgartner, S., *et al.* (1987) Genes & Dev. 1, 1247-1267). Partial sequence data from genomic clones revealed a 0.9 kb XhoI fragment with open reading frames showing homology to the mammalian $p70^{S6K}$ catalytic domain. This fragment is used to screen a oligo-dT primed Canton S embryonic cDNA library in the UniZap XR vector (Stratagene). Analysis of 11 independent cDNAs revealed all are internally primed from a polyadenine stretch at base 2062 which also occurs in the genomic sequence. Thus, a genomic clone containing the ORF extending 3' of these adenines is used to screen the cDNA library mentioned above as well as a random primed Canton S embryonic cDNA library in the Stratagene lambda ZAPII vector (provided by Dr. Carl Thummel). A variable number of adenine residues is noted in clones from the random primed library at position 2062, most likely arising due to enzyme stuttering during cDNA synthesis. Overlapping clones make up a transcription unit of 3.6 kb with multiple consensus polyadenylation signals, two of which are utilised in recovered clones. Comparison of genomic and cDNA sequences 3' of base 2062 revealed two apparent strain polymorphisms resulting in substitution of Leu for Phe632 and insertion of three Glu residues at amino acid 567 in the genomic sequence. DNA sequencing is carried out using specific oligonucleotide primers and the Bst (Bio-Rad) and Sequenase version 2.0 (U.S. Biochemical Corp.) systems. The *Drosophila* $p70^{S6K}$ DNA sequence is submitted to GenBank/EMBL/ DNA Data Bank of Japan under the accession number U 66562.

Plasmid Constructs and Cell Transfections.

A construct containing the entire *Drosophila* p70$^{S6K}$ coding region is generated by fusing a HindIII/KpnI genomic fragment to the HindIII site at base 2026 within the cDNA and a KpnI site within the polylinker of a cloning vector. PCR mutagenesis employing PFU polymerase and specific oligonucleotides is used to insert a myc-epitope tag at the amino-terminus of the *Drosophila* p70$^{S6K}$ cDNA. This construct is confirmed by sequencing and subcloned into the vector pBD1119 under the control of the alpha tubulin promoter (Theurkauf, W.E., *et al.* (1986) Proc. Natl. Acad. Sci. USA 83, 8477-8481) and transfected into Schneider line 2 (S2) cells using a modified calcium phosphate-precipitation method (Chen, C.A. & Okayama, H. (1988) Biotechniques 6, 632-638).

Antibody Production and Immunoblot Analysis.

The peptide MADVSDPSELFDLELHDLEY coupled to a carboxy terminal tyrosine is conjugated to Keyhole Limpet hemocyanin and used to produce a rabbit polyclonal antiserum (Neosystem). The peptide is coupled to CNBr activated Sepharose (Pharmacia) and used to affinity purify specific antibodies after ammonium sulphate precipitation of the antiserum (Harlow, H.A. & Lane, D. (1988) Antibodies - A laboratory manual; Cold Spring Harbor Lab. Press, Cold Spring Harbor, NY). Affinity-purified antibody (D20 antibody) is diluted 1/1000 for Western blot analysis. Extracts from S2 cells, prepared as described (Tuta, M., *et al.* (1989) Cell 57, 817-824), are subjected to Western blot analysis with the D20 antibody. Blots are decorated with a horse radish peroxidase conjugated anti-rabbit secondary antibody and bands detected using the ECL system (Amersham Corp).

Kinase Assays and Immunoprecipitations.

Cell extracts are prepared and *Drosophila* p70$^{S6K}$ activity is measured as previously described (28) or following immunoprecipitation (Lane, H.A., *et al.* (1992) EMBO J 11, 1743-1749) with the D20 antibody (2.5μg).

Mono Q Chromatography.

S2 cells are plated at 5x107 cells per 15cm dish in Schneider's medium supplemented with antibiotic and 10% foetal calf serum. After 24h, cells are changed to Schneider's medium without serum and after an additional 36h, 5 plates of c lls are treated with 100μM

cycloheximide for two hours with or without pretreatment with 20nm rapamycin for 15min. Cell extracts are prepared by dounce homogenisation in Extraction buffer lacking PNPP as described in Reinhard *et al.*, above. Briefly, each extract is centrifuged for 30min at 3x105g, filtered through a 0.2μm membrane (Millipore), and applied at a flow rate of 0.5ml per min to a 1 ml Mono Q column (Pharmacia) equilibrated in Buffer F (Kozma, S.C., *et al.* (1989) EMBO J 8, 4125-4132). The column is washed with 10ml of Buffer F, developed at 0.5ml per min with a 30ml gradient from 0-0.5M NaCl in the same buffer, and each fraction is diluted 20-fold and assayed for kinase activity. Factions 16-19 are concentrated on 100μl of FFQ Sepharose and eluted with 300μl of 0.5M NaCl in Buffer F.

Expression mutant analysis.

Genomic DNA flanking the left insertion point of the P element in fs(3)07084 was cloned by plasmid rescue as described (Pirrotta, V. in Drosophila: a practical approach (ed. D.B. Roberts) IRL Press, Oxford, UK, 1986)) and sequenced using an oligonucleotide primer based on the P element inverted repeats (Rubin, G.M. & Spradling, A.C. Drosophila Nucleic Res. 11, 6341-6351 (1983)) and *Drosophila* $p70^{S6K}$ specific primers. Genomic DNA was isolated and subjected to Southern blot analysis as previously described (Lindsley, D.J. & Zimm, G.G. Academic Press, San Diego, Calif. USA. (1992)). Canton S was used as the wild type strain. All markers and Balancer chromosomes are as described (Wieschaus, E. & Nesslein-Volhard, C. in Drosphila: a practical approach (ed D.B. Roberts) IRL Press, Oxford, UK. 1986)).

Eggs were collected and mounted in Hoyers mountant as described. Microscopy was done with a Zeiss Axiophot.

Ovaries were dissected from 3-4 day old Canton S (wild type) or fs(3)*Drosophila* $p70^{S6K}$/fs(3)*Drosophila* $p70^{S6K}$ females, immediately frozen in N2 and then stored at -800C. Total protein was extracted by dounce homogenisation of ovaries in extraction buffer (Lane, H.A. et al. EMBO J. 11, 1743-1749 (1992)) containing in addition 25μm leupeptin, 10mm EDTA, 10mm EGTA and 5 mg/ml aprotinin. Proteins were separated by 12% SDS-PAGE and subjected to Western blot analysis with a 1/50 dilution of D20 antibody followed by an HRP conjugated secondary antibody and bands were visualised using ECL (Boehringer). Total RNA was isolated from ovaries and Northern blot analysis performed. For rRNA analysis, eggs were collected every two hr. and k pt on ice in PBS until 30 eggs were accumulated, approximately 6 hr. The ggs were then gently pelleted by centrifugation, the

supernatant removed, 200 µl of lysis buffer 30 containing 0.1% SDS was added, the eggs homogenised in an Eppendorf tube with a Pellet Pestle ( Kontes), the extract heated to 60°C for 1 min., followed by centrifugation at maximum speed for 15 min. on an Eppendorf centrifuge to remove insoluble material , and 150 µl of the extract loaded on a 4ml isokinetic sucrose gradient and analysed at A260 as previously described (Thomas, G. et al. Exp. Cell. Res. 108, 253-258 (1977)). The amount of RNA present was calculated from the area of the 28S and 18S rRNA peaks.

RESULTS

Drosophila $p70^{S6K}$.

To identify cDNA clones encoding the Drosophila $p70^{S6K}$ homologue, Drosophila genomic clones are first isolated by hybridisation with a rat $p70^{S6K}$ cDNA probe, and are then used to screen Drosophila cDNA libraries (see Materials & Methods). A number of overlapping cDNA fragments are isolated which, when aligned, represent a transcription unit of 3.6 kb. Conceptual translation of this cDNA, termed *Drosophila* $p70^{S6K}$ , shows it contains a single large open reading frame encoding a protein of 637 amino-acids with a predicted M.W. 73 kD, containing all the conserved motifs found in Ser/Thr kinases. Northern blot analysis of total RNA revealed three transcripts of 2.8 kb, 3.7 kb and 5.0 kb in size. The 2.8 kb transcript is significantly more abundant in females than males, whereas in males the 3.7 kb transcript is most abundant. The size of the hypothetical *Drosophila* $p70^{S6K}$ cDNA is consistent with the 3.7 kb message seen by Northern blot analysis. Both the 2.8 kb and 5.0 kb transcripts may represent either alternatively-spliced transcripts or mRNAs which utilise distinct polyadenylation signals (see Materials and Methods). All three transcripts appear specific for the *Drosophila* $p70^{S6K}$ gene, as a probe derived from outside of the *Drosophila* $p70^{S6K}$ catalytic domain detected identical transcripts (data not shown). Although multiple transcripts may be indicative of a gene family, the majority of Drosophila genes are single copy and the mammalian $p70^{S6K}$ is represented by a single gene (Reinhard, C., *et al.* (1992) Proc. Natl. Acad. Sci. USA 89, 4052-4056). To determine the number of Drosophila $p70^{S6K}$ gene copies, a high stringency genomic Southern blot analysis is carried out. Only three r striction fragments are detected in XhoI-dig sted genomic DNA when *Drosophila* $p70^{S6K}$ cDNA is employed as a probe, consistent with the presenc of two

XhoI sites within the *Drosophila* p70$^{S6K}$ cDNA. Furthermore, double digestion of genomic DNA with EcoRI and XhoI does not reveal additional fragments. Depending on the specific restriction enzyme used, the *Drosophila* p70$^{S6K}$ cDNA hybridised to only two or three genomic DNA fragments indicating an approximate size of 15 kb for the gene excluding potential regulatory regions. These results argue for a single copy gene and are consistent with *Drosophila* p70$^{S6K}$ representing the unique member of this family.

Conservation of Regulatory Domains.

Analysis of the predicted *Drosophila* p70$^{S6K}$ amino-acid sequence shows 57% overall identity to the mammalian p70$^{S6K}$ sequence with the highest homology in the catalytic domain where the identity is 78% and the similarity is 86%. The acidic amino-terminal domain of mammalian p70$^{S6K}$ confers rapamycin sensitivity on the kinase (Weng, Q-P., *et al.* (1995) Mol. Cell. Biol. 15, 2333-2340; Dennis, P.B., *et al.* (1996)). In *Drosophila* p70$^{S6K}$ this domain is also highly acidic, displaying 55% similarity with its mammalian counterpart, suggesting it may play an equivalent role in *Drosophila* p70$^{S6K}$ . Immediately adjacent to the catalytic domain, *Drosophila* p70$^{S6K}$ contains a 68 amino acid long sequence which has a striking 72% identity with its mammalian homologue. In mammalian p70$^{S6K}$ this domain couples the catalytic and autoinhibitory domains, and is termed the linker region. It has recently been noted that this region is conserved in many members of the second messenger subfamily of Ser/Thr kinases and may play a critical role in regulating kinase activity (Pearson, R.B., *et al.* (1995) EMBO J. 14, 5279-5287). This linker region is immediately followed by the putative autoinhibitory domain (see below) which contains four Ser/Thr-Pro sites whose phosphorylation is thought to relieve the inhibitory impact of this domain as well as contribute to full kinase activation. Consistent with this model it is previously shown that peptides covering this domain, residues 400 to 432, inhibit kinase activity in the low μM range (Price, D.J., *et al.* (1991) J. Biol. Chem. 266, 16281-16284; Flotow, H. & Thomas, G. (1992) J. Biol. Chem. 267, 3074-3078). Strikingly, in *Drosophila* p70$^{S6K}$ the sequence R417SPRRTPR425 immediately following the linker region is very similar in context to a piece of the mammalian autoinhibitory domain, R410SPRRFIGSPR419. Although this peptide only represents a fragment of the autoinhibitory domain found in p70$^{S6K}$, in r cent studies it is defined as the sequence within the larger peptide which is responsible for ex rting the inhibitory effect on p70$^{S6K}$. Thus in *Drosophila* p70$^{S6K}$ this sequence may be involved in auto-regulation of kinase activity in a

manner similar to that predicted in mammalian p70$^{S6K}$. The final stretch of amino acids, residues 425 to 637, display no significant homology with the mammalian p70$^{S6K}$ nor with known proteins from database searches, indicating this carboxy-terminal domain is unique to *Drosophila* p70$^{S6K}$. However, this domain contains a highly basic lysine residues beginning at amino-acid 525, which is separated by thirty five amino acids from a long stretch of acidic amino acids nearer the carboxy-terminus. Given that there is no detectable nuclear targeting sequence in the *Drosophila* p70$^{S6K}$ equivalent to that found in the mammalian p85s6k isoform, it would be of interest to test whether these sequences perform a similar function in *Drosophila* p70$^{S6K}$.

*Drosophila* p70$^{S6K}$ expression.

To determine whether the *Drosophila* p70$^{S6K}$ cDNA encodes a protein product equivalent to the endogenous protein, Drosophila Schneider line 2 (S2) cells are transiently transfected with a *Drosophila* p70$^{S6K}$ construct containing a myc epitope-tag at its amino-terminus (Materials and Methods). Following transfection, protein products are visualised by Western blot analysis, employing the monoclonal antibody 9E10 (Ming, X.F., *et al.* (1994) Nature 371, 426-429). A protein band with Mr 70k is specifically detected in extracts from transiently transfected cells, but is absent in extracts from control cells. To determine whether an equivalent protein could be detected in extracts of S2 cells, a rabbit polyclonal antibody (D20) is generated against a peptide representing amino acids 1-19 of the *Drosophila* p70$^{S6K}$ amino-terminus (Materials and Methods). This antibody specifically recognises an endogenous protein of Mr 70k in S2 cells which co-migrates with the kinase produced ectopically from transient transfection of the cDNA in S2 cells that is detected with either the D20 antiserum or the 9E10 antibody. Longer exposures of the film do not reveal additional bands that might be suggestive of an isoform equivalent to the mammalian p85s6k (Reinhard, C., *et al.* (1994) EMBO J. 13, 1557-1565). Preincubation of the D20 antiserum with the antigenic peptide prevents binding to *Drosophila* p70$^{S6K}$. Thus, the *Drosophila* p70$^{S6K}$ cDNA encodes a protein which is antigenically equivalent to a Drosophila protein which migrates at a similar molecular weight.

S6 kinase activity and sensitivity to rapamycin.

Activation of the rat p70$^{S6K}$ is associated with phosphorylation of ten residues (Pearson, R.B., *et al.* (1995) EMBO J. 14, 5279-5287), five of which are conserved in

*Drosophila* p70$^{S6K}$. By mutational analysis, three of the ten sites, T229, S371 and T389, have been argued to be critical for p70$^{S6K}$ activation. These sites are conserved in *Drosophila* p70$^{S6K}$ as T238, S380, and T398. In addition the principal target of rapamycin-induced p70$^{S6K}$ dephosphorylation and inactivation is T389, with T229 acting as a secondary target. However, for rapamycin to exert this inhibitory response on mammalian p70$^{S6K}$, the macrolide requires the acidic residues at the amino-terminus. As discussed above this may be a feature also conserved in *Drosophila* p70$^{S6K}$ . To examine whether S2 cells contain S6 kinase activity and if so, whether this activity is sensitive to rapamycin, mammalian 40S ribosomes are incubated with extracts from S2 cells treated with cycloheximide, an agent known to activate mammalian p70$^{S6K}$ (Kozma, S.C., *et al.* (1989) EMBO J 8, 4125-4132; Mukhopadhayay, N.K., *et al.* (1992) J. Biol. Chem. 267, 3325-3335), in the absence or presence of the macrolide. Mammalian 40S ribosomes are employed in these assays as they are found to be as good a substrate as their Drosophila counterparts (data not shown). Cycloheximide treatment of S2 cells increased S6 kinase activity 2.2 fold over basal levels and rapamycin pretreatment prevented this increase, reducing activity below basal levels. However, a significant amount of rapamycin insensitive kinase activity towards S6 is still apparent.

To distinguish between these rapamycin sensitive and insensitive S6 kinase activities and to determine whether either represented *Drosophila* p70$^{S6K}$ , extracts from cycloheximide-stimulated S2 cells, pretreated with or without rapamycin, are fractionated by Mono Q chromatography. The fractions eluted from the Mono Q column are monitored by A280 and subjected to an in vitro S6 kinase assay. Two peaks of S6 kinase activity emerged from the column . The first peak contained less S6 kinase activity, eluted at 0.13M NaCl and is rapamycin insensitive. The second peak of activity eluted at 0.29M NaCl, contained approximately 15-fold more S6 kinase activity, and is completely abolished by rapamycin pretreatment. To assess whether the S6 kinase activity in this peak is attributable to *Drosophila* p70$^{S6K}$ , we used FFQ Sepharose to concentrate the fractions 16 to 18 containing the rapamycin sensitive S6 kinase activity. The concentrated samples from cells treated with cycloheximide either in the absence or presence of rapamycin pretreatment, are either immunoprecipitated and assayed for S6 kinase activity or analysed on Western blots employing the D20 antibody. The antibody specific for *Drosophila* p70$^{S6K}$ immunoprecipitated S6 kinase activity from the sample stimulated with cycloheximide, but no activity could be detected from the sample pretreated with rapamycin prior to the addition

of cycloheximide. Western blot analysis of thes fractions revealed a slower electrophoretic mobility on SDS PAGE for the active *Drosophila* p70$^{S6K}$ as compared to the inactive kinase. This altered electrophoretic mobility is similar to the mobility shift induced by phosphorylation of the mammalian p70$^{S6K}$, an effect that is ablated by rapamycin treatment. Thus, cycloheximide treatment induces the activation of *Drosophila* p70$^{S6K}$, which is apparently regulated by phosphorylation and is rapamycin sensitive.

Expression Mutant.

We have localised *Drosophila* p70$^{S6K}$ by in situ hybridisation to chromosome position 64F1-3 (data not shown) with the aim of determining whether mutants existed in the gene and, if so, whether they exhibit a phenotype consistent with the presumptive role of p70$^{S6K}$ in ribosome biogenesis. A search of FlyBase11 revealed that several P element insertion mutants map to this location. By RFLP analysis one of these mutants, fs(3)07084 (Karpen, G.H. & Spradling A.C. Genetics 132, 737-753 (1992); Spradling, A.C. *et al.* Proc. Natl. Acad. Sci. USA 92, 10824-10830 (1995)) is found to contain a P element in the 5' region of the *Drosophila* p70$^{S6K}$ gene. Sequencing of flanking genomic DNA, localised the P element insertion to 41 base pairs upstream of the *Drosophila* p70$^{S6K}$ AUG initiator codon, strongly implying that the mutation would disrupt *Drosophila* p70$^{S6K}$ expression (see below).

Homozygous flies eventually eclose as adults, however their final number is roughly a quarter of the expected value (Table 1A). These flies emerge as adults after an average delay of approximately four days as compared with either their heterozygous or wild type counterparts (Table 1A). This represents a relative 30% reduction in the rate of development as compared with heterozygous or wild type flies (Table 1A). When homozygous mutant males are crossed with wild type females there is no discernible effect on development, whereas eggs from a reciprocal cross failed to complete embryogenesis. Analysis of ovaries of 2 to 3 day old homozygous females appear mature and exhibit no obvious defects (data not shown). However, homozygous mutant females lay an average of five times less eggs than either heterozygous or wild type females (Table 1B). These eggs display aberrant, antler-like, dorsal appendages which do not complete development relative to dorsal appendages of wild type eggs. Consistent with this finding the dorsal-most follicle cells have not elongated and the eggs appear comparatively smaller, exhibiting a short egg phenotype. The dorsal appendage is an extension of th chorion, and though synthesised by the follicle cells, is d pendent on th growth and longation of the egg

chamber that takes place between stages 11-13 of oogenesis (Spradling, A.C. in The Development of Drosophila melanogaster (eds Bate, M. & Martinez Arias, A.) Vol. 1, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1993). This step of development immediately follows the dramatic upregulation of ribosome biogenesis, stages 8-10, which is required for the completion of oogenesis (Mermod, J-J. *et al.* Development Bio. 57, 393-402 (1977)). Indeed many of the gene products required for the massive growth of the oocyte between stages 11-13 are produced in large amounts during stage 10B.

A P element mobilisation scheme led to recovery of the wild type phenotype, therefore we have termed this gene *Drosophila* p70$^{S6K}$ fs(3)07084. This finding suggested that the P element insertion led to a reduction of kinase expression, similar to that reported for other genes in which the P element is inserted in the 5'UTR of the gene. To examine this possibility we compared the levels of *Drosophila* p70$^{S6K}$ from ovaries of wild type flies with those found in heterozygous and homozygous mutants by western blot analysis employing a specific antibody. The results show that the amount of *Drosophila* p70$^{S6K}$ is notably reduced in heterozygous females and that it is not detectable in homozygous mutants, suggesting that this mutant is a null for *Drosophila* p70$^{S6K}$ . However, Northern blot analysis of total RNA from ovaries of homozygous mutant females, employing the *Drosophila* p70$^{S6K}$ cDNA as a probe, revealed three transcripts. The relative amounts of these transcripts are significantly less than in wild type females and they migrate with a relative increased molecular weight. This difference in size suggests that the mutant transcripts initiate transcription from within the P element. Genetic support for *Drosophila* p70$^{S6K}$ presence comes from P element excisions in which the transposon is incorrectly removed, such that no viable homozygous flies are recovered. In addition, heterozygous females flies carrying *Drosophila* p70$^{S6K}$fs(3)07084 on one allele and a deficiency spanning the *Drosophila* p70$^{S6K}$ on the other allele, DF(3L)ZN4711, lay even less nonviable eggs than homozygous *Drosophila* p70$^{S6K}$fs(3)07084 females. Thus the P element insertion appears to strongly suppress expression of *Drosophila* p70$^{S6K}$ , allowing the observation of the phenotype, which would not have been perceived in the absence of the gene.

The slower rate of development observed for homozygous *Drosophila* p70$^{S6K}$ fs(3)07084 flies as well as the exhibited decrease in fertility and egg size are characteristic of Minute and bobbed mutations. The Minute loci comprise a class of approximately fifty haplo-insuffic nt loci, which are speculated to affect ribosomal proteins (Vaslet, C.A. et al. Nature 285, 674-676 (1980)). To date, five Minute loci have been demonstrated to represent

deletions of ribosomal proteins of both subunits (Vaslet, C.A. et al. Nature 285, 674-676 (1980)). The bobbed mutants affect rRNA genes20 with the severity of the phenotype proportional to the number of deleted functional genes (Shermoen, A.W. & Kiefer, B.I. Cell 4, 275-280 (1975)). Flies mutant for either of these two classes of genes display reduced fertility, exhibit a retarded rate of development, and in some cases display small slender bristles. Though the latter phenotype is not observed in *Drosophila* $p70^{S6K}$ fs(3)07084 homozygotes, the other characteristics are highly similar, indicating a reduction in the rate of translation due to a decrease in ribosome content. Stages 8-10 of Drosophila oogenesis are thought to represent the most spectacular example of ribosome synthesis of any tissue at any time of development, ie most of the $5 \times 10^{10}$ ribosomes produced in the nurse cells and oocyte are generated within this period (Kay, M.A. & Jacobs-Lorena, M. TIG 3, 347-351 (1987)). These ribosomes are required for the dramatic growth and development of the oocyte at the end of oogenesis. Accompanying this event is the selective translational upregulation of ribosomal protein mRNA transcripts, many of which contain a 5'TOP5. Given the putative role of the $p70^{S6K}$ in mediating the selective upregulation of these mRNAs at the translational level, we reasoned that the *Drosophila* $p70^{S6K}$ fs(3)07084 phenotype may be explained by a reduction in ribosome content. To examine this possibility, we isolated total ribosomes from eggs laid by wild type or homozygous mutant females and scored for the amount of rRNA as a measure of ribosome content. Quantitation of these results revealed a striking 40% reduction in both 40S and 60S subunits in eggs derived from mutant females versus their wild type counterparts.

The observed phenotypic manifestations of reduced *Drosophila* $p70^{S6K}$ expression are consistent with the predicted role of the kinase in protein synthesis (Jefferies, H.B.J. & Thomas, G. in Translational Control (eds Hershey, J.W.B.Mathews, M.B. & Sonenberg, N.) Vol. 1, Cold Spring Harbor Press, Cold Spring Harbor, 1995)) and emphasise the importance of the $p70^{S6K}$ signalling pathway during cell growth and development. The function of the $p70^{S6K}$ signalling pathway in these processes is beginning to be elucidated5, as are the identity of putative upstream signalling molecules (Downward, J. Nature 376, 553-554 (1995)). The availability of a mutant which cripples but does not ablate this pathway will prove immensely valuable in unravelling the functional importance of potential regulatory domains within the kinase and the relative importance of downstream targets predicted to mediate its biological responses.

TABLE 1

A. Offspring of heterozygous matings

| Flies | Number of Adult Flies | Eclosion |
|---|---|---|
| homozygotes | 93 (357 expected) | 16.3 days |
| heterozygotes | 715 | 12.7 days |

B. Eggs Laid

| Flies | Eggs per day | Eggs counted |
|---|---|---|
| wild-type | 37.7 | 2208 |
| heterozygotes | 40.4 | 5630 |
| homozygotes | 8.52 | 1388 |

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: NOVARTIS AG
(B) STREET: Schwarzwaldallee
(C) CITY: Basel
(E) COUNTRY: Switzerland
(F) POSTAL CODE (ZIP): 4002
(G) TELEPHONE: +41 61 696 11 11
(H) TELEFAX: + 41 61 696 79 76
(I) TELEX: 962 991

(ii) TITLE OF INVENTION: Kinase

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2556 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Drosophila melanogaster

(vii) IMMEDIATE SOURCE:
(B) CLONE: Drosophila p70S6K

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:474..2384

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CGCAAACAAA GAAAAAAGTG CATTTTAATT ACTGATTACT GCATTGCGAT TAGGCGTTCG      60

CGAACAACAA CGTTTTCTTG CACATCAGCG GACTCAAAGG GCGCGCGTGG GCAGGGCATT     120

GAGCACAGTG TCTGTGTGCG TGAGTGTGGG TGCGTTGTGC GTGCGTCGCA GTGTTGGTGC     180

GTGTGCCCCC ATGTGTGTGT GTATTTGTAT TGCCATTAAC TAATAAACAA TAACCTTCGG     240

GCCACGACGA AAGCCACGAA ATCGGCACCG CTGCTGTTCT GTTCTGTACT GTTCCATCTG     300

AACCAACCAG CTCAACAAGA GGACTCATAG CCAGAGACTA AAATAAAACG CCAACGCGCT     360

TCCGCCCCCC CCGCGAAAGG AGCGCCCAGT TCAGGAGCGG ACCAGCGGCG ATCCACTTCC     420

CATATCCATA TCCAAACAGA ATCGCACTCG CCCAAAACAC ACACACGCAC GCA ATG         476
                                                          Met
                                                           1

GCG GAC GTG AGC GAT CCC AGC GAA CTG TTC GAC CTG GAG CTG CAC GAC         524
Ala Asp Val Ser Asp Pro Ser Glu Leu Phe Asp Leu Glu Leu His Asp
              5                  10                  15

CTG GAA CTG CAG GAC GAC AAG GCC AGG GAC TCC GAC GAC GAC AGG ATC         572
Leu Glu Leu Gln Asp Asp Lys Ala Arg Asp Ser Asp Asp Asp Arg Ile
         20                  25                  30

GAG CTG GAC GAC GTT GAC CTA GAA CCG GAA TTG TGT ATT AAT CTG CAC         620
Glu Leu Asp Asp Val Asp Leu Glu Pro Glu Leu Cys Ile Asn Leu His
     35                  40                  45
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CAA GAC ACT GAG GGC CAG GAG ACC ATA CAG CTC TGC GAG GAG AAT GTT        668
Gln Asp Thr Glu Gly Gln Glu Thr Ile Gln Leu Cys Glu Glu Asn Val
 50                  55                  60                  65

AAT CCA GGT AAA ATC AAG CTG GGA CCC AAG GAC TTT GAG CTC AAG AAG        716
Asn Pro Gly Lys Ile Lys Leu Gly Pro Lys Asp Phe Glu Leu Lys Lys
                 70                  75                  80

GTC CTT GGC AAA GGC GGT TAT GGC AAA GTA TTT CAG GTG CGC AAG ACC        764
Val Leu Gly Lys Gly Gly Tyr Gly Lys Val Phe Gln Val Arg Lys Thr
             85                  90                  95

GCT GGA CGA GAT GCT AAC AAA TAT TTT GCC ATG AAG GTG CTC AAA AAG        812
Ala Gly Arg Asp Ala Asn Lys Tyr Phe Ala Met Lys Val Leu Lys Lys
        100                 105                 110

GCA TCC ATT GTG ACC AAT CAA AAG GAC ACA GCG CAC ACC CGC GCC GAG        860
Ala Ser Ile Val Thr Asn Gln Lys Asp Thr Ala His Thr Arg Ala Glu
    115                 120                 125

CGC AAT ATA CTC GAG GCA GTC AAG CAT CCC TTC ATA GTG GAG CTA GTT        908
Arg Asn Ile Leu Glu Ala Val Lys His Pro Phe Ile Val Glu Leu Val
130                 135                 140                 145

TAT GCC TTC CAG ACA GAC GGA AAA CTA TAC CTT ATA CTT GAA TAT CTC        956
Tyr Ala Phe Gln Thr Asp Gly Lys Leu Tyr Leu Ile Leu Glu Tyr Leu
                150                 155                 160

AGC GGT GGA GAG CTG TTC ATG CAT TTG GAG CGT GAG GGC ATC TTC TTA       1004
Ser Gly Gly Glu Leu Phe Met His Leu Glu Arg Glu Gly Ile Phe Leu
            165                 170                 175

GAG GAT ACC ACA TGC TTC TAT CTA AGC GAA ATC ATT TTG GCC TTG GGC       1052
Glu Asp Thr Thr Cys Phe Tyr Leu Ser Glu Ile Ile Leu Ala Leu Gly
        180                 185                 190

CAT CTA CAC AAA CTG GGC ATC ATC TAC CGC GAT CTG AAG CCC GAG AAC       1100
His Leu His Lys Leu Gly Ile Ile Tyr Arg Asp Leu Lys Pro Glu Asn
    195                 200                 205

ATA CTG CTC GAT GCA CAG GGA CAT GTG AAG CTC ACG GAC TTT GGA CTG       1148
Ile Leu Leu Asp Ala Gln Gly His Val Lys Leu Thr Asp Phe Gly Leu
210                 215                 220                 225
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TGC AAG GAG CAC ATA CAA GAG GGT ATT GTC ACC CAC ACC TTC TGC GGC      1196
Cys Lys Glu His Ile Gln Glu Gly Ile Val Thr His Thr Phe Cys Gly
                230                 235                 240

ACA ATT GAG TAC ATG GCA CCT GAA ATT TTG ACC AGA AGT GGC CAT GGC      1244
Thr Ile Glu Tyr Met Ala Pro Glu Ile Leu Thr Arg Ser Gly His Gly
            245                 250                 255

AAA GCA GTC GAC TGG TGG TCA CTG GGC GCT CTC ATG TTT GAC ATG CTC      1292
Lys Ala Val Asp Trp Trp Ser Leu Gly Ala Leu Met Phe Asp Met Leu
        260                 265                 270

ACA GGA GTC CCA CCC TTC ACC GCC GAG AAT CGC AAG AAG ACC ATC GAG      1340
Thr Gly Val Pro Pro Phe Thr Ala Glu Asn Arg Lys Lys Thr Ile Glu
    275                 280                 285

ACC ATT CTG AAA GCC AAG CTC AAT CTG CCA GCC TAC CTC ACA CCG GAA      1388
Thr Ile Leu Lys Ala Lys Leu Asn Leu Pro Ala Tyr Leu Thr Pro Glu
290                 295                 300                 305

GCC AGG GAT CTG GTG CGT CGC CTG ATG AAG CGG CAG GAA CCT CAG CGC      1436
Ala Arg Asp Leu Val Arg Arg Leu Met Lys Arg Gln Glu Pro Gln Arg
                310                 315                 320

CTT GGC AGC GGA CCC GAG GAT GCG GCG GCT GTT CAA ATA CAC CCA TTC      1484
Leu Gly Ser Gly Pro Glu Asp Ala Ala Ala Val Gln Ile His Pro Phe
            325                 330                 335

TTC AAA CAC GTC AAC TGG GAC GAT GTG CTC GCC AGA CGC CTC GAG CCG      1532
Phe Lys His Val Asn Trp Asp Asp Val Leu Ala Arg Arg Leu Glu Pro
        340                 345                 350

CCT ATA AAA CCG CTC TTG AGA AGC GAG GAT GAT GTC TCA CAG TTC GAT      1580
Pro Ile Lys Pro Leu Leu Arg Ser Glu Asp Asp Val Ser Gln Phe Asp
    355                 360                 365

ACA AGA TTC ACA AGA CAA ATT CCA GTG GAT TCC CCT GAT GAT ACA ACG      1628
Thr Arg Phe Thr Arg Gln Ile Pro Val Asp Ser Pro Asp Asp Thr Thr
370                 375                 380                 385
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CTA AGC GAA AGT GCC AAT TTA ATT TTC CAA GGT TTC ACC TAC GTT GCA      1676
Leu Ser Glu Ser Ala Asn Leu Ile Phe Gln Gly Phe Thr Tyr Val Ala
                390                 395                 400

CCC TCG ATA CTG GAG GAT ATG CAT CGG GCC AAC CGG ATG CCA GCA CGC      1724
Pro Ser Ile Leu Glu Asp Met His Arg Ala Asn Arg Met Pro Ala Arg
            405                 410                 415

TCC CCA CGA CGC ACT CCA CGC CAG CTG CCG GAC AGC AGC TTC CGC CTG      1772
Ser Pro Arg Arg Thr Pro Arg Gln Leu Pro Asp Ser Ser Phe Arg Leu
        420                 425                 430

CAG TTC CCA TCG GCC AAT GTG GGC GCC AAT GCG CCT GCT GGC CAT GCA      1820
Gln Phe Pro Ser Ala Asn Val Gly Ala Asn Ala Pro Ala Gly His Ala
    435                 440                 445

CGG TCA TTC GCA GCG ATC CGG GAT GTT TGC ACG AGC CAC GCC GCC GCA      1868
Arg Ser Phe Ala Ala Ile Arg Asp Val Cys Thr Ser His Ala Ala Ala
450                 455                 460                 465

TCA CAT GCA GAC ATT TGC GCC GCG TCC ATC GCC GGC GCA GGA CGA GAT      1916
Ser His Ala Asp Ile Cys Ala Ala Ser Ile Ala Gly Ala Gly Arg Asp
                470                 475                 480

GAT GGA CGT GCA GGG TCT GCC GAT GGT CTA AAG GCT GGA GCG ATT GCT      1964
Asp Gly Arg Ala Gly Ser Ala Asp Gly Leu Lys Ala Gly Ala Ile Ala
            485                 490                 495

TCC CAA CCA ACC AAC CCA TCC CAT CCC GTC CGT TCC GTC GCA ACC CAG      2012
Ser Gln Pro Thr Asn Pro Ser His Pro Val Arg Ser Val Ala Thr Gln
        500                 505                 510

CAA CTG CAT CAT AAG CTT CCT CTC CCT ACC CCA AAA AAA AAA AAA AAA      2060
Gln Leu His His Lys Leu Pro Leu Pro Thr Pro Lys Lys Lys Lys Lys
    515                 520                 525

AAA AAA AAA CAG CAA AAG ATT ACA GAA ATT GAC AGT TAC TAT TAT GAC      2108
Lys Lys Lys Gln Gln Lys Ile Thr Glu Ile Asp Ser Tyr Tyr Tyr Asp
530                 535                 540                 545

GTA TGT GGA GTG TGG ACT TGG TTA AGG ATA CGG AAT GAG CAG GAC GAT      2156
Val Cys Gly Val Trp Thr Trp Leu Arg Ile Arg Asn Glu Gln Asp Asp
                550                 555                 560
```

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CAT CAG GAG GTG GCG GAG GAG GAG GAG GAG GAG GAG GAG GAG GCT GAG      2204
His Gln Glu Val Ala Glu Glu Glu Glu Glu Glu Glu Glu Ala Glu
            565                 570                 575

CAG CAC GAG GAG CAC ATG ACC TCA GTG CGC GAA ATT GTT TTT GTT AAA      2252
Gln His Glu Glu His Met Thr Ser Val Arg Glu Ile Val Phe Val Lys
        580                 585                 590

GAA AAG CGC GCT CGG ATC GCG CTT TTC GAT GTT TAT GAT TAT GAG AAT      2300
Glu Lys Arg Ala Arg Ile Ala Leu Phe Asp Val Tyr Asp Tyr Glu Asn
    595                 600                 605

GAT TAT GAA TAT GAT TAT GAT TAT GAG GCA GAC GGA GAA GAT GAT TGT      2348
Asp Tyr Glu Tyr Asp Tyr Asp Tyr Glu Ala Asp Gly Glu Asp Asp Cys
610                 615                 620                 625

GCT ACG AGA AGG AAA GCG TTC GTT TTT GGA TAC ACC TAGATACTAC          2394
Ala Thr Arg Arg Lys Ala Phe Val Phe Gly Tyr Thr
                630                 635

AGTTAGAGAC ATCCACATAA GCATATGCTA TAGCAATTAC TATATACATA CACCTAGAGA    2454

GATGGTTACC CGACCCGGAT CCCAACAGCC CCAAAAACCT ATCCGTGTTT ATGTATAAAG    2514

ATTATACTTA CACTTATGTT TTATATAGGC AAAAAGGTTA AC                      2556
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 637 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Ala Asp Val Ser Asp Pro Ser Glu Leu Phe Asp Leu Glu Leu His
  1               5                  10                  15

Asp Leu Glu Leu Gln Asp Asp Lys Ala Arg Asp Ser Asp Asp Asp Arg
            20                  25                  30
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Ile Glu Leu Asp Asp Val Asp Leu Glu Pro Glu Leu Cys Ile Asn Leu
35 40 45

His Gln Asp Thr Glu Gly Gln Glu Thr Ile Gln Leu Cys Glu Glu Asn
50 55 60

Val Asn Pro Gly Lys Ile Lys Leu Gly Pro Lys Asp Phe Glu Leu Lys
65 70 75 80

Lys Val Leu Gly Lys Gly Gly Tyr Gly Lys Val Phe Gln Val Arg Lys
85 90 95

Thr Ala Gly Arg Asp Ala Asn Lys Tyr Phe Ala Met Lys Val Leu Lys
100 105 110

Lys Ala Ser Ile Val Thr Asn Gln Lys Asp Thr Ala His Thr Arg Ala
115 120 125

Glu Arg Asn Ile Leu Glu Ala Val Lys His Pro Phe Ile Val Glu Leu
130 135 140

Val Tyr Ala Phe Gln Thr Asp Gly Lys Leu Tyr Leu Ile Leu Glu Tyr
145 150 155 160

Leu Ser Gly Gly Glu Leu Phe Met His Leu Glu Arg Glu Gly Ile Phe
165 170 175

Leu Glu Asp Thr Thr Cys Phe Tyr Leu Ser Glu Ile Ile Leu Ala Leu
180 185 190

Gly His Leu His Lys Leu Gly Ile Ile Tyr Arg Asp Leu Lys Pro Glu
195 200 205

Asn Ile Leu Leu Asp Ala Gln Gly His Val Lys Leu Thr Asp Phe Gly
210 215 220

Leu Cys Lys Glu His Ile Gln Glu Gly Ile Val Thr His Thr Phe Cys
225 230 235 240

Gly Thr Ile Glu Tyr Met Ala Pro Glu Ile Leu Thr Arg Ser Gly His
245 250 255

Gly Lys Ala Val Asp Trp Trp Ser Leu Gly Ala Leu Met Phe Asp Met
260 265 270

Leu Thr Gly Val Pro Pro Phe Thr Ala Glu Asn Arg Lys Lys Thr Ile
275 280 285

Glu Thr Ile Leu Lys Ala Lys Leu Asn Leu Pro Ala Tyr Leu Thr Pro
290 295 300

Glu Ala Arg Asp Leu Val Arg Arg Leu Met Lys Arg Gln Glu Pro Gln
305 310 315 320

Arg Leu Gly Ser Gly Pro Glu Asp Ala Ala Ala Val Gln Ile His Pro
325 330 335

Phe Phe Lys His Val Asn Trp Asp Asp Val Leu Ala Arg Arg Leu Glu
340 345 350

Pro Pro Ile Lys Pro Leu Leu Arg Ser Glu Asp Asp Val Ser Gln Phe
355 360 365

Asp Thr Arg Phe Thr Arg Gln Ile Pro Val Asp Ser Pro Asp Asp Thr
370 375 380

Thr Leu Ser Glu Ser Ala Asn Leu Ile Phe Gln Gly Phe Thr Tyr Val
385 390 395 400

Ala Pro Ser Ile Leu Glu Asp Met His Arg Ala Asn Arg Met Pro Ala
405 410 415

Arg Ser Pro Arg Arg Thr Pro Arg Gln Leu Pro Asp Ser Ser Phe Arg
420 425 430

Leu Gln Phe Pro Ser Ala Asn Val Gly Ala Asn Ala Pro Ala Gly His
435 440 445

Ala Arg Ser Phe Ala Ala Ile Arg Asp Val Cys Thr Ser His Ala Ala
450 455 460

Ala Ser His Ala Asp Ile Cys Ala Ala Ser Ile Ala Gly Ala Gly Arg
465 470 475 480

Asp Asp Gly Arg Ala Gly Ser Ala Asp Gly Leu Lys Ala Gly Ala Ile
485 490 495

Ala Ser Gln Pro Thr Asn Pro Ser His Pro Val Arg Ser Val Ala Thr
500 505 510

Gln Gln Leu His His Lys Leu Pro Leu Pro Thr Pro Lys Lys Lys Lys
515 520 525

Lys Lys Lys Lys Gln Gln Lys Ile Thr Glu Ile Asp Ser Tyr Tyr Tyr
530 535 540

Asp Val Cys Gly Val Trp Thr Trp Leu Arg Ile Arg Asn Glu Gln Asp
545 550 555 560

Asp His Gln Glu Val Ala Glu Glu Glu Glu Glu Glu Glu Glu Glu Ala
565 570 575

Glu Gln His Glu Glu His Met Thr Ser Val Arg Glu Ile Val Phe Val
580 585 590

Lys Glu Lys Arg Ala Arg Ile Ala Leu Phe Asp Val Tyr Asp Tyr Glu
595 600 605

Asn Asp Tyr Glu Tyr Asp Tyr Asp Tyr Glu Ala Asp Gly Glu Asp Asp
610 615 620

Cys Ala Thr Arg Arg Lys Ala Phe Val Phe Gly Tyr Thr
625 630 635

Claims:

1. *Drosophila* $p70^{S6K}$ or a derivative thereof.

2. *Drosophila* $p70^{S6K}$ or a derivative thereof having an amino acid sequence derivable from SEQ ID No. 2.

3. A nucleic acid sequence encoding *Drosophila* $p70^{S6K}$ or a derivative thereof according to claim 1 or claim 2.

4. A *Drosophila melanogaster* fly in which $p70^{S6K}$ expression has been modulated.

5. An expression system comprising a nucleotide sequence encoding *Drosophila* $p70^{S6K}$ and appropriate control elements.

6. Use of a fly according to claim 4 for determining the effect of an agent or mixture of agents on $p70^{S6K}$ activity.

7. A method for screening an agent or mixture of agents for an effect on $p70^{S6K}$ activity comprising exposing a *Drosophila melanogaster* fly to said agent or mixture of agents and assessing the biological activity thereof.

8. Method according to claim 7 wherein the biological activity is measured by observing oogenesis in the fly.

9. A transgenic *Drosophila melanogaster* fly in which the biological activity of $p70^{S6K}$ is modified, optionally by transforming the fly with a gene encoding a $p70^{S6K}$ modulator or disrupting the expression of such a gene.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/54, 9/12, A01K 67/033, C12Q 1/48, C07K 16/40 — A3

(11) International Publication Number: WO 98/03662

(43) International Publication Date: 29 January 1998 (29.01.98)

(21) International Application Number: PCT/EP97/03680

(22) International Filing Date: 11 July 1997 (11.07.97)

(30) Priority Data:
9615498.4 24 July 1996 (24.07.96) GB

(71) Applicant (*for all designated States except US*): NOVARTIS AG [CH/CH]; Schwarzwaldallee 215, CH-4058 Basel (CH).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): STEWART, Mary [US/CH]; Birkenstrasse 7, CH-4123 Allschwil (CH). KOZMA, Sara [BE/FR]; (FR). THOMAS, George [US/FR]; 4, Grand Rue, F-68220 Ranspach-le-Haut (FR).

(74) Agent: ROTH, Bernhard, M.; Novartis AG, Patent- und Markenabteilung, Lichtstrasse 35, CH-4002 Basel (CH).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**

*With international search report.*

*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(88) Date of publication of the international search report:
12 March 1998 (12.03.98)

(54) Title: DROSOPHILA MELANOGASTER P70 S6 KINASE

(57) Abstract

The invention provides *Drosophila melanogaster*$p70^{S6K}$, as well as nucleic acids encoding this kinase. The sequence of *Drosophila* $p70^{S6K}$ and the gene encoding it are represented in SEQ ID NO. 2 and 1 respectively. The invention moreover provides mutated forms of *Drosophila* $p70^{S6K}$, including constitutively active and dominant negative forms thereof, which are useful in the study of $p70^{S6K}$ activity. Furthermore, the invention provides expression systems which produce *Drosophila* $p70^{S6K}$ in *Drosophila* and other organisms, and in particular systems in which expression of *Drosophila* $p70^{S6K}$ has been modulated so as to facilitate the study of its activity.

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# INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 97/03680

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6 C12N15/54 C12N9/12 A01K67/033 C12Q1/48 C07K16/40

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No |
|---|---|---|
| Y | J. RUSSELL GROVE ET AL.: "Cloning and expression of two human p70 S6 kinase polypeptides differing only at their amino termini." MOLECULAR AND CELLULAR BIOLOLGY, vol. 11, no. 11, November 1991, pages 5541-5550, XP002051876 see the whole document --- | 1-3,5 |
| Y | H.A. LANE ET AL.: "Identification and early activation of a Xenopus laevis p70 s6k following progesterone-induced meiotic maturation." THE EMBO JOURNAL, vol. 11, no. 5, 1992, pages 1743-1749, XP002051877 see the whole document --- | 1-3,5 |

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[X] Further documents are listed in the continuation of box C

[ ] Patent family members are listed in annex

° Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 13 January 1998 | 28/01/1998 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Hix, R |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No |
|---|---|---|
| Y | B. HARMANN ET AL.: "cDNA encoding a 59kDa homolog of ribosomal protein S6 kinase from rabbit liver." FEBS LETTERS, vol. 273, no. 1,2, October 1990, pages 248-252, XP002051878 see the whole document --- | 1-3,5 |
| Y | P. BANERJEE ET AL.: "Molecular structure of a major insulin/mitogen-activated 70-kDa S6 protein kinase." PROC. NATL. ACAD. SCI. USA., vol. 87, November 1990, pages 8550-8554, XP002051879 see the whole document --- | 1-3,5 |
| Y | S.C. KOZMA ET AL.: "Cloning of the mitogen-activated S6 kinase from rat liver reveals an enzyme of the second messenger subfamily." PROC. NATL. ACAD. SCI. USA, vol. 87, October 1990, pages 7365-7369, XP002051880 see the whole document --- | 1-3,5 |
| Y | S. FERRARI ET AL.: "Activation of p70 s6k is associated with phophorylation of four clustered sites displaying Ser/Thr-Pro motifs." PROC. NATL. ACAD. SCI. USA, vol. 89, August 1992, pages 7282-7286, XP002051881 cited in the application see the whole document --- | 1-3,5 |
| P,X | M.J. STEWART ET AL.: "The Drosophila p70 s6k homolog exhibits conserved regulatory elements and rapamycin sensitivity." PROC. NATL. ACAD. SCI. USA, vol. 93, October 1996, pages 10791-10796, XP002051882 see the whole document --- | 1-9 |
| P,X | K.L. WATSON ET AL.: "A Drosphila gene structurally and functionally homologous to the mammalian 70-kDa S6 kinase gene." PROC. NATL. ACAD. SCI. USA, vol. 93, November 1996, pages 1394-13698, XP002051883 see the whole document --- -/-- | 1-9 |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | R.B. PEARSON ET AL.: "The principle target of rapamycin-induced p70 s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain." THE EMBO JOURNAL, vol. 14, no. 21, 1995, pages 5279-5287, XP000561164 cited in the application see figure 6 --- | |
| A | J. CHUNG ET AL.: "PDGF- and insulin-dependent pp70 s6k activation mediated by phosphatidylinositol-3-OH kinase." NATURE, vol. 370, 7 July 1994, pages 71-75, XP002051885 cited in the application see the whole document ----- | |

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : G01N 33/50, A61K 49/00, C12N 15/01, A01K 67/033 — A1

(11) International Publication Number: **WO 00/37938**

(43) International Publicati n Date: 29 June 2000 (29.06.00)

(21) International Application Number: PCT/IB99/02020

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(30) Priority Data:
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(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 09/217,694 (CIP)
Filed on 21 December 1998 (21.12.98)

(71) Applicant *(for all designated States except US)*: THE GENETICS COMPANY, INC. [CH/CH]; Universität Zürich, Winterhurerstrasse 190, CH-8057 Zurich (CH).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: HAFEN, Ernst [CH/CH]; Hochstrasse 95, CH-8044 Zürich (CH).

(74) Agent: SCHLICH, George, William; Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: FUNCTION-BASED SMALL MOLECULAR WEIGHT COMPOUND SCREENING SYSTEM IN *DROSOPHILA MELANOGASTER*

(57) Abstract

A methodology for screening libraries of compounds for desirable biological/therapeutic activities in a system that may be automated for the microinjection of compound(s) of interest into the open circulatory system (i.e., hemolymph) of *Drosophila* larvae genetically modified to sensitize a particular biochemical pathway, such as those related to a human disease, either by expression of a human disease gene or by the activation of a *Drosophila* gene that in the adult fly results in the development of an easily detectable phenotypes such that compounds that selectively interfere with this specific biochemical pathway will modify or suppress the phenotype and can be identified rapidly and efficiently.

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# INTERNATIONAL SEARCH REPORT

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| | ational Application No |
| | PCT/IB 99/02020 |

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7 G01N33/50 A61K49/00 C12N15/01 A01K67/033

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N A61K C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 98 03662 A (CIBA GEIGY AG ;STEWART MARY (CH); THOMAS GEORGE (FR); KOZMA SARA ()<br>29 January 1998 (1998-01-29)<br>the whole document | 1-3,15 |
| X | US 5 593 862 A (FENG GUOPING ET AL)<br>14 January 1997 (1997-01-14)<br>column 3, line 30 -column 4, line 10;<br>claims; example 14 | 15 |
| X | WO 92 14817 A (IMP CANCER RES TECH)<br>3 September 1992 (1992-09-03)<br>the whole document | 1-6,9,10 |

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[X] Further documents are listed in the continuation of box C.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 9 May 2000 | 24/05/2000 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Luzzatto, E |

1

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

| I. ...tional Application No |
|---|
| PCT/IB 99/02020 |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | KAUFFMANN R.C. ET AL.: "Activated Drosophila Ras1 is selectively suppressed by isoprenyl transferase inhibitors" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 92, November 1995 (1995-11), pages 10919-10923, XP002137250 US the whole document | 1-10, 12-15 |
| X | BISHOP J.G. ET AL.: "Expression of an activated ras gene causes developmental abnormalities in transgenic Drosophila melanogaster" GENES AND DEVELOPMENT (ISSN: 0890-9369), vol. 2, no. 5, 1988, pages 567-577, XP000886242 the whole document | 15 |
| X | DICKSON B.J. ET AL: "Mutations modulating Raf signaling in Drosophila eye development" GENETICS, vol. 142, 1996, pages 163-171, XP000886247 US the whole document | 15 |
| A | THOMPSON J.N. ET AL. : "Modification of cell growth and longevity using an in vivo assay in Drosophila melanogaster" GRWTH (ISSN 0017-4793), vol. 48, no. 1, 1984, pages 86-92, XP000086250 the whole document | 1 |
| P,X | WO 99 37672 A (BAYLOR COLLEGE OF MEDICINE) 29 July 1999 (1999-07-29) the whole document | 1-3,15 |

1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 99/02020

| Patent document cited in search report | Publication date | Patent family member(s) | Publication dat |
|---|---|---|---|
| WO 9803662 A | 29-01-1998 | AU 4113897 A<br>EP 0915982 A | 10-02-1998<br>19-05-1999 |
| US 5593862 A | 14-01-1997 | US 5871940 A | 16-02-1999 |
| WO 9214817 A | 03-09-1992 | NONE | |
| WO 9937672 A | 29-07-1999 | AU 2342899 A | 09-08-1999 |

# .TENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

# PCT

To:
SCHLICH, George, William
Attn. RITTER, Stephen David
Mathys & Squire
100 Gray's Inn Road
London WC1X 8AL
UNITED KINGDOM



NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

| | |
|---|---|
| | Date of mailing *(day/month/year)* 24/05/2000 |
| Applicant's or agent's file reference 18111-001 | **FOR FURTHER ACTION** See paragraphs 1 and 4 below |
| International application No. PCT/IB 99/02020 | International filing date *(day/month/year)* 16/12/1999 |
| Applicant THE GENETICS COMPANY INC. et al. | |

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

**Filing of amendments and statement under Article 19:**
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

**When?** The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

**Where?** Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

**For more detailed instructions,** see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** *The applicant is reminded of the following:*

*Shortly after* **18 months** *from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.*

*Within* **19 months** *from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).*

*Within* **20 months** *from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.*

| | |
|---|---|
| Name and mailing address of the International Searching Authority<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL-2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Authorized officer<br>Véronique Baillou |

Form PCT/ISA/220 (July 1998)

PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

| Applicant's or agent's file reference | FOR FURTHER ACTION | see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below. |
|---|---|---|
| 18111-001 | | |
| International application No. | International filing date *(day/month/year)* | (Earliest) Priority Date *(day/month/year)* |
| PCT/IB 99/02020 | 16/12/1999 | 21/12/1998 |
| Applicant | | |
| THE GENETICS COMPANY INC. et al. | | |

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

# NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

## INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

**What parts of the international application may be amended?**

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

**When?**

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

**Where not to file the amendments?**

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been /is filed, see below.

**How?**

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

**The amendments must be made in the language in which the international application is to be published.**

**What documents must/may accompany the amendments?**

**Letter (Section 205(b)):**

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

**The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.**

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

**The following examples illustrate the manner in which amendments must be explained in the accompanying letter:**

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

**"Statement under article 19(1)" (Rule 46.4)**

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

**It must be in the language in which the international appplication is to be published.**

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

**Consequence if a demand for international preliminary examination has already been filed**

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

**Consequence with regard to translation of the international application for entry into the national phase**

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

# PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

## PCT

### NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

| | |
|---|---|
| **Date of mailing** (day/month/year)<br>18 August 2000 (18.08.00) | |
| **International application No.**<br>PCT/IB99/02020 | **Applicant's or agent's file reference**<br>GWS/22028 |
| **International filing date** (day/month/year)<br>16 December 1999 (16.12.99) | **Priority date** (day/month/year)<br>21 December 1998 (21.12.98) |
| **Applicant**<br>HAFEN, Ernst | |

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

17 July 2000 (17.07.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

| | |
|---|---|
| **The International Bureau of WIPO**<br>**34, chemin des Colombettes**<br>**1211 Geneva 20, Switzerland** | Authorized officer<br>Juan Cruz |
| Facsimile No.: (41-22) 740.14.35 | Telephone No.: (41-22) 338.83.38 |

# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

| Applicant's or agent's file reference | FOR FURTHER ACTION | see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below. |
|---|---|---|
| 18111-001 | | |
| International application No. | International filing date *(day/month/year)* | (Earliest) Priority Date *(day/month/year)* |
| PCT/IB 99/02020 | 16/12/1999 | 21/12/1998 |
| Applicant | | |
| THE GENETICS COMPANY INC. et al. | | |

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

# INTERNATIONAL SEARCH REPORT

International Application No
[redacted]TB 99/02020

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7 G01N33/50 A61K49/00 C12N15/01 A01K67/033

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N A61K C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 98 03662 A (CIBA GEIGY AG ;STEWART MARY (CH); THOMAS GEORGE (FR); KOZMA SARA ()<br>29 January 1998 (1998-01-29)<br>the whole document | 1-3,15 |
| X | US 5 593 862 A (FENG GUOPING ET AL)<br>14 January 1997 (1997-01-14)<br>column 3, line 30 -column 4, line 10;<br>claims; example 14 | 15 |
| X | WO 92 14817 A (IMP CANCER RES TECH)<br>3 September 1992 (1992-09-03)<br>the whole document | 1-6,9,10 |

-/--

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 9 May 2000 | 24/05/2000 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31–70) 340–2040, Tx. 31 651 epo nl,<br>Fax: (+31–70) 340–3016 | Luzzatto, E |

1

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International Application No
[redacted]IB 99/02020

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | KAUFFMANN R.C. ET AL.: "Activated Drosophila Ras1 is selectively suppressed by isoprenyl transferase inhibitors" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 92, November 1995 (1995-11), pages 10919-10923, XP002137250 US the whole document | 1-10, 12-15 |
| X | BISHOP J.G. ET AL.: "Expression of an activated ras gene causes developmental abnormalities in transgenic Drosophila melanogaster" GENES AND DEVELOPMENT (ISSN: 0890-9369), vol. 2, no. 5, 1988, pages 567-577, XP000886242 the whole document | 15 |
| X | DICKSON B.J. ET AL: "Mutations modulating Raf signaling in Drosophila eye development" GENETICS, vol. 142, 1996, pages 163-171, XP000886247 US the whole document | 15 |
| A | THOMPSON J.N. ET AL. : "Modification of cell growth and longevity using an in vivo assay in Drosophila melanogaster" GRWTH (ISSN 0017-4793), vol. 48, no. 1, 1984, pages 86-92, XP000086250 the whole document | 1 |
| P,X | WO 99 37672 A (BAYLOR COLLEGE OF MEDICINE) 29 July 1999 (1999-07-29) the whole document | 1-3,15 |

1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 99/02020

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9803662 A | 29-01-1998 | AU 4113897 A | 10-02-1998 |
| | | EP 0915982 A | 19-05-1999 |
| US 5593862 A | 14-01-1997 | US 5871940 A | 16-02-1999 |
| WO 9214817 A | 03-09-1992 | NONE | |
| WO 9937672 A | 29-07-1999 | AU 2342899 A | 09-08-1999 |

PATENT COOPERATION TREATY

PCT

REC'D 17 JAN 2001

WIPO PCT

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

| Applicant's or agent's file reference | FOR FURTHER ACTION | See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) |
|---|---|---|
| GWS/22028 | | |
| International application No. | International filing date *(day/month/year)* | Priority date *(day/month/year)* |
| PCT/IB99/02020 | 16/12/1999 | 21/12/1998 |
| International Patent Classification (IPC) or national classification and IPC<br>G01N33/50 | | |
| Applicant<br>THE GENETICS COMPANY INC. et al. | | |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

| Date of submission of the demand | Date of completion of this report |
|---|---|
| 17/07/2000 | 12.01.2001 |
| Name and mailing address of the international preliminary examining authority:<br>European Patent Office<br>D-80298 Munich<br>Tel. +49 89 2399 - 0 Tx: 523656 epmu d<br>Fax: +49 89 2399 - 4465 | Authorized officer<br>Luzzatto, E<br>Telephone No. +49 89 2399 8169 |

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IB99/02020

## I. Basis of th report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
**Description, pages:**

1-27 as originally filed

**Claims, No.:**

1-17 as originally filed

**Drawings, sheets:**

1/3-3/3 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Yes: | Claims | 11,16,17 |
| | No: | Claims | 1-10,12-15 |
| Inventive step (IS) | Yes: | Claims | |
| | No: | Claims | 11,16,17 |
| Industrial applicability (IA) | Yes: | Claims | 1-17 |
| | No: | Claims | |

2. Citations and explanations
**see separate sheet**

**VI. Certain documents cited**

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

**see separate sheet**

**VII. Certain defects in the international application**

The following defects in the form or contents of the international application have been noted:
**see separate sheet**

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
**see separate sheet**

**Re Item V**

**Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

Reference is made to the following documents:

D1: WO-A-9803662
D2: WO-A-9214817
D3: P.N.A.S.,92,10919-23,95
D4: US-A-5593862
D5: Genetics, 142, 163-171, 96
D6: Growth, 48, 86-92, 84
D7: WO-A-9937672

1) The present application does not meet the requirements of Art. 33(1) and (2) PCT due to lack of novelty.

1.1) D1 (see p. 2, l. 3-p. 3, l. 6, p. 20, l. 32-p. 21, l. 6, claims) describes the use of a transgenic Drosophila carrying a mutated $p70^{S6K}$ gene and its use in a screening assay. It thus anticipates the subject-matter of claims **1-3 and 15**.

1.2) D2 (see p. 3, l. 23-p.4, l. 25, p. 18, l. 1-19, ex. 1 and 2, claims) disclose transgenic Drosophila expressing a cytochrome P450 reductase and its use in the characterisation of compounds. The compounds are injected into third instar larvae. Hence, D2 anticipates the subject-matter of claims **1-6, 9, 10, 12, 13 and 15.**

1.3) D3 (see abstract, p. 10919, right-hand col., l. 6-48, "Materials and Methods", "Concluding Remarks") describes a peptide screening assay comprising the use of a transgenic Drosophila expressing a mutated *ras1*. The effect of the drug, which was injected into the third-instar larvae, was assessed by morphological analysis of the eye. D3 thus anticipates the subject-matter of claims **1-10, 12-15**.

1.4) D4 (see passages cited in the Search Report) discloses transgenic Drosophila expressing a mutated cation channel protein and its use in screening assays.

It thus anticipates the subject-matter of claims **1-3 and 15**.

1.5) D5 discloses a transgenic Drosophila carrying an activated *raf* gene (see abstract and results). It thus anticipates the subject-matter of claim **15**.

1.6) D6 discloses a drug screening assay comprising the use of a Drosophila mutant. The drug effect is assessed by phenotype analysis (see abstract, table 1 and discussion). D6, thus, anticipates claims **1-3 and 15**.

2) Claims 11, 16 and 17 relate to features which are commonplace in the art which, in any case, do not appear to exhibit any unexpected effect and are thus devoid of an inventive step (Art. 33(3) PCT).

**Re Item VI**
**Certain documents cited**

1) D7 has been published the 29/7/99 and claims a priority date of 26/1/98. It is not part of the state of the art according to R. 64(1) PCT (R. 64.3 PCT). It could however become relevant to the issue of novelty in the national or regional phase and to that of inventive step as well, if the priority should not be valid.

**Re Item VII**
**Certain defects in the international application**

1) Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1-D6 is not mentioned in the description, nor are these documents identified therein.

**Re Item VIII**
**Certain observations on the international application**

1) Claims 14 and 15 contravene Art. 5 and 6 PCT because their subject-matter is not sufficiently disclosed in the description which only provides examples relating to the *raf* gene. In view of the unrelatedness of the genes listed in the said claims to

each other, the fact that the method can work with *raf* does not suffice to indicate that any other of the said genes would work as well.

2) Claims 14 and 15 are unclear (Art. 6 PCT) because the genes to which they relate are not clearly defined because:
- the terms "pathway", "system", "and the like" are vague;
- some terms do not relate to genes (e.g. "nitric oxide", "lipid metabolites").